universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

The role of dynamin during polarity establishment in *C. elegans* one-cell embryos

angestrebter akademischer Grad

Magister/Magistra der Naturwissenschaften (Mag. rer.nat.)

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Wien, am 11.08.2008

Summary

One-cell *C. elegans* embryos establish the anterior-posterior polarity axis shortly after fertilization. Polarity establishment depends on a cue from the sperm-derived centrosomes, which subsequently leads to a change in acto-myosin activity in one half of the embryo. Centrosome-cortex proximity during polarity establishment in one-cell *C. elegans* embryos is paralleled in several other cell polarization events, specifically in T cell polarization and hippocampal axon specification. In both of these polarized cells, vesicle trafficking plays an integral role in polarization. I therefore wondered whether vesicle trafficking also plays an essential role in polarity establishment in *C. elegans* embryos. To study this question, I am taking advantage of a previously isolated temperature-sensitive allele of dynamin, a GTPase required for vesicle formation. Using quantitative time-lapse microscopy, I am analyzing the effects of dynamin inactivation at different times during polarity establishment. My initial experiments suggest that dynamin is required immediately prior to or during polarity initiation. This is similar to the temporal requirement for centrosomes. In some cases, dynamin is needed for a short time after initiation of polarity establishment. I am continuing to investigate the mechanism by which dynamin contributes to polarity establishment by analyzing the effect of dynamin inactivation on various components of the polarity establishment pathway. Therefore I generated worm strains expressing markers for specific components of the vesicle transport pathway. These strains and existing GFP lines, e.g. RAB-5::GFP, were crossed to the temperature sensitive dynamin strain to investigate the behavior of the endomembrane system in the absence of dynamin function. Overexpression of GFP::PAR-3, a marker of anterior polarity, in embryos lacking dynamin function led to more severe defects during polarity establishment than in the dynamin mutant alone. This might result from the change in the ratio of anterior and posterior PAR proteins, suggesting that dynamin could work in parallel to PAR-2, a posterior polarity protein. This idea is supported by experiments showing that dynamin mutant embryos depleted of PAR-2 have more severe polarity establishment defects than dynamin mutants alone. To further investigate this topic, I looked at the polarity establishment phenotypes of embryos depleted of other vesicle transport proteins by RNAi or treated with specific vesicle transport inhibitors.

Zusammenfassung

C. elegans Embryonen im Einzellstadium legen die anterior-posterior Polaritätsachse bereits kurze Zeit nach der Befruchtung fest. Die Entwicklung der Zellpolarität hängt von den Zentrosomen ab, welche vom Spermium zur Verfügung gestellt werden. Dies führt zu einer Veränderung der Aktivität des Aktin-Myosin-Netzwerkes in einer Hälfte des Embryos. Die Nähe zwischen Zentrosom und Cortex während der Entwicklung der Zellpolarität in *C. elegans* Einzellembryonen tritt auch in vielen anderen Zellpolarisierungen auf. Darunter wären zum Beispiel die T-Zellpolarisierung und die Spezifizierung hippokampischer Axone. In beiden dieser Beispiele spielt der intrazelluläre Vesikeltransport eine wesentliche Rolle für die Polarisierung der Zelle. Ich habe mich daher gefragt, ob der intrazelluläre Vesikeltransport auch in der Polaritätsentwicklung in *C. elegans* Embryonen eine grundlegende Rolle spielt. Für meine Experimente benützte ich ein bereits isoliertes temperatur-sensitives Allel von Dynamin, einer GTPase, die für die Vesikelbildung verantwortlich ist. Mit Hilfe von quantitativer Time-Lapse-Mikroskopie werden die Folgen der Inaktivierung von Dynamin zu bestimmten Zeitpunkten während der Entwicklung der Zellpolarität analysiert. Meine anfänglichen Experimente deuten darauf hin, dass Dynamin kurz vor oder während der Einleitung der Polaritätsentwicklung gebraucht wird, ähnlich wie die Zentrosomen. Manchmal jedoch scheint es als ob Dynamin auch noch einige Zeit nach der Einleitung der Zellpolarisierung gebraucht wird. Weitere Experimente sollten Aufschluss über den Effekt der Inaktivierung von Dynamin auf verschiedene Komponenten des Signalweges geben, der zur Entwicklung der Zellpolarität führt. Dafür wurden verschiedene neue Wurmstämme durch Kreuzungen generiert, die ein GFP-getaggtes Protein und das temperatursensitive Allel von Dynamin enthalten. Die GFP-getaggten Proteine sind bestimmte Marker für Komponenten des Vesikeltransportwegs, zum Beispiel RAB-5::GFP als Marker für frühe Endosomen. Manche der GFP-Stämmen standen bereits zur Verfügung, andere wurden durch Klonierungen und Wurmbombardierung erst neu hergestellt. Die mögliche Überexpression von PAR-3, einem Marker für anteriore Polarität, in den PAR-3::GFP Würmern ohne Dynamin führte zu einer stärkeren Ausprägung des Phenotyps gegenüber den temperatur-sensitiven Würmern alleine. Dies könnte möglicherweise durch die Veränderung des anterior-posterior PAR Proteinverhältnisses verursacht werden. In diesem Fall wäre es mögich, dass Dynamin parallel zu PAR-2, einem posterioren Polaritätsprotein, arbeitet. Diese Hypothese wird durch par-2 RNAi Experimente noch verstärkt, denn sie ergaben einen ähnlichen Phenotyp wie PAR-3::GFP in temperatur-sensitiven Würmern. Um noch näher auf das Thema

einzugehen, machte ich weitere Versuche mit RNAi von anderen Vesikeltransportproteinen und behandelte Embryonen mit speziellen Vesikeltransportinhibitoren.

Content

1 Introduction

1.1 Cell polarity

Most cell types have some form of polarity, such that different regions are created in the cell, which can carry out distinct functions. A clear example is epithelial cells in the intestine. The specialized protein distribution in different regions makes it possible to import nutrients at the apical side of the cell and transport them out at the basolateral side to finally release them into the blood. Neuronal cells are also polarized, which allows the directed release of synaptic vesicles and the transmission of signals.

While neurons and intestinal epithelial cells utilize cell polarity to carry out post-mitotic functions, other cells utilize cell polarity to create diversity during mitosis. Stem cells divide into two different daughter cells, one is equal to the stem cell and the other becomes a cell that differentiates into a specified cell type. Cell polarity is required for the asymmetric division of stem cells. Similar asymmetric cell divisions generate diversity during embryonic development. A representative model organism for this is the nematode *Caenorhabditis elegans.* Asymmetric cell divisions after fertilisation are fundamental to specify the different cellular lineages that will give rise to the whole worm. Hence the establishment of cell polarity is indispensable (Sulston et al., 1983).

There must be a certain cue that makes cells polarize. The signals can be of various types; communication between cells, chemoattractants and even light, but the result is in any case very similar, the formation of distinct domains on the cell cortex (Gonczy and Hyman, 1996; Nelson, 2003).

1.2 Asymmetric cell division

In *C. elegans* embryos, a series of four asymmetric cell divisions creates the six different founder cells that are necessary to form a functional organism (Sulston et al., 1983). During this process the three main body axes of *C. elegans* are also formed. After the symmetry-breaking event the first anterior-posterior axis forms. During the following divisions the dorso-ventral axis and the left-right axis form (Gonczy and Rose, 2005).

There are indications that the symmetry breaking is caused by the centrosomes, which are donated by the sperm (Cowan and Hyman, 2004a, b).

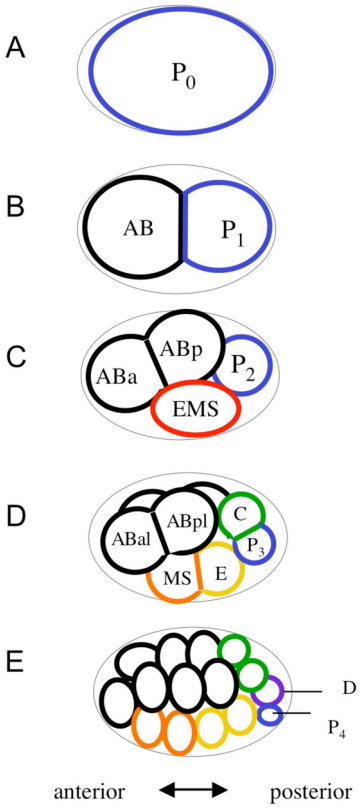


Figure 1: In 5 asymmetric cell divisions, 6 different types of cells are created in the embryo, called founder cells. The blue cells will later become the germ-line; anterior (left), posterior (right), dorsal (up) and ventral (down) (Gönczy, P. and Rose, 2005; http://www.wormbook.org).

The centrosomes are situated close to the cortex of the embryo at the time of polarity establishment. They transmit a signal to the cortex, which leads to the initiation of polarity. After this initial event the posterior domain can expand independently of the centrosomes.

1.3 Polarity Establishment

1.3.1 Polarity establishment in *C. elegans* occurs on three different levels in the cell

1. The first step is the acto-myosin reorganization. Following fertilization, a cross-linked acto-myosin network is distributed all over the cell cortex. Due to the contractility of the network, there are cortical ingressions over the whole cell surface, called ruffling. The symmetry-breaking event of polarity establishment results in a progressive disassembly of the network on one side of the embryo, near the side of sperm entry. This side becomes the posterior side of the embryo. The acto-myosin network regresses until it occupies half of the embryo. This side becomes the anterior. The other side, the posterior, is non-

contractile (Gonczy and Rose, 2005). There is a deep ingression at the boarder between the posterior and the anterior domain during polarity establishment, which is more stable than the other ruffles. This is called the pseudocleavage furrow (Cowan and Hyman, 2004a).

Figure 2: cortical acto-myosin network in the one-cell embryo. Outline of *C. elegans* one-cell embryo (grey) with acto-myosin network (orange). The pseudocleavage furrow is situated at the boarder between the anterior (left) and posterior (right) half of the embryo.

This process is partially mediated by Rho family GTPases. Active Rho is required for the contractility of the cortex (Jenkins *et al.*, 2006; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). RhoGEF (guanidine nucleotide exchange factor) ECT-2 and RhoGAP (GTPase-activating protein) CYK-4 are responsible for the activation and inactivation of Rho, respectively (Glotzer, 2005). During polarity establishment, contractility appears to be regulated locally and there is specific suppression on the posterior side of the embryo through asymmetric localization of ECT-2 and CYK-4 (Cowan and Hyman, 2007).

2. In parallel to the reorganization of the acto-myosin network, the anterior and posterior PAR domains are established. Initially the anterior PAR proteins, including PAR-3, PAR-6 and PKC-3, are spread around the whole cell cortex. Simultaneous with the acto-myosin network, the anterior PAR proteins retract toward the anterior side of the cell until they occupy the contractile half of the embryo (Cuenca *et al.*, 2003; Etemad-Moghadam *et al.*, 1995; Hung and Kemphues, 1999; Tabuse *et al.*, 1998). The posterior PAR proteins, PAR-1 and PAR-2, appear on the smooth side of the cell (Boyd *et al.*, 1996; Cuenca *et al.*, 2003; Guo and Kemphues, 1995). There is likely an antagonism between the anterior and posterior PAR proteins as they are never co-localized in wild type embryos (Cowan and Hyman, 2004a).

   Various other proteins play a role in the establishment and maintenance of the par domains. Among them there is the small G protein CDC-42, which interacts with PAR-6 (Gotta and Ahringer, 2001). It is possible that CDC-42 modulates the acto-myosin network and thereby prevents NMY-2 (non muscle myosin) to go to the posterior side of the embryo (Gonczy and Rose, 2005).

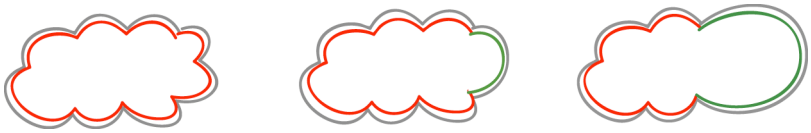


Figure 3**:** Establishment of PAR-protein domains in *C. elegans* one-cell embryo. Anterior PAR complex consisting of PAR-3, PAR-6 and PKC-3 indicated in red, posterior PAR proteins, PAR-1 and PAR-2 shown in green. Anterior is to the left, posterior to the right.

3. Cytoplasmic polarization is the third step in polarity establishment. Proteins in the cytosol, fate determinants and other proteins that appear to mediate their localization, are segregated unequally to one side of the cell in response to spatial information established by the PAR domains.

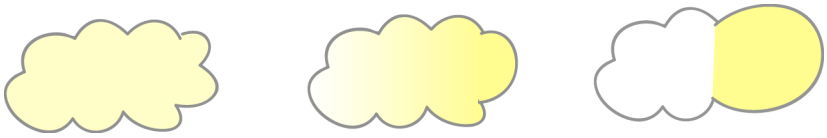


Figure 4: cytoplasmic polarity establishment in *C. elegans* one-cell embryo

The cell fate determinants include for instance PIE-1 and PAL-1 (Gonczy and Rose, 2005). Among the mediators of asymmetric fate determinant localization are proteins like MEX-1 (Guedes and Priess, 1997; Mello *et al.*, 1992), MEX-5/6 (Schubert *et al.*, 2000), POS-1 (Tabara et al., 1999), MEX-3 (Draper *et al.*, 1996) and SPN-4 (Gomes *et al.*, 2001). The anterior and posterior PAR domains also control the asymmetric distribution of organelles and macro-molecular structures within the cytoplasm, including the mitotic spindle (see section 1.3.2) and endosomes (see section1.4.1.1).

1.3.2 Spindle positioning

After polarity establishment is complete, the female pronucleus moves towards the male pronucleus. The male pronucleus and the associated centrosomes also start moving, and the two pronulei meet each other in the posterior half of the cell. After pronuclear meeting the pronuclei and the associated centrosomes move to the middle of the embryo while rotating 90°. This event is followed by nuclear envelope breakdown. Afterwards the spindle forms and, upon anaphase onset, elongates to segregate sister chromatids. The spindle moves toward the posterior half of the embryo by unequal pulling forces acting on astral microtubules at the anterior and posterior cortices (Gonczy and Rose, 2005). The posterior displacement of the spindle results in a corresponding displacement of the cytokinesis furrow, generating daughter cells of different sizes.

The asymmetric distribution of the pulling forces is under the control of the PAR proteins (Grill et al., 2001).

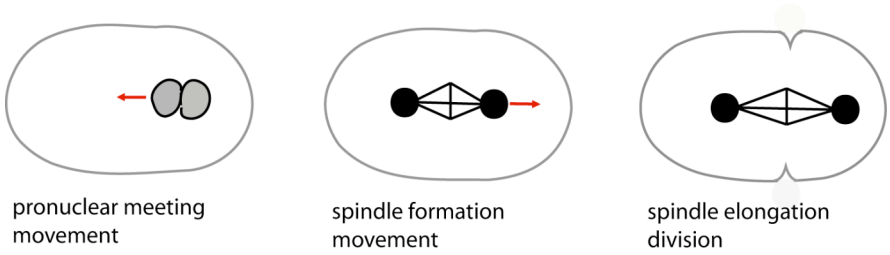


Figure 5: Schema of an embryo during spindle positioning. The picture on the left shows pronuclear meeting and the movement of the pronulcei towards the cell centre. The middle picture shows the spindle that elongates and moves towards the posterior pole of the embryo. In the right picture there is the elongated spindle and the ingressions of the cortex, which indicates the initiation of cytokinesis. Anterior is to the left, posterior to the right; pronuclei are grey circles, centrosomes are black circles and the spindle is indicated by black lines.

1.4 Vesicle Trafficking

1.4.1 Intracellular vesicle transport

Intracellular vesicle transport consists of two main routs of transport, endocytosis and exocytosis (secretion).

Vesicle transport is essential not only for the adult organism, but also during development. It is essential for the delivery of proteins to the cell membrane or to the external environment as well as the internalization of extracellular molecules. Thus vesicle transport underlies how a cell exchanges information with its environment. Cell-cell signalling can be enhanced or down regulated by certain kinds of vesicle trafficking.

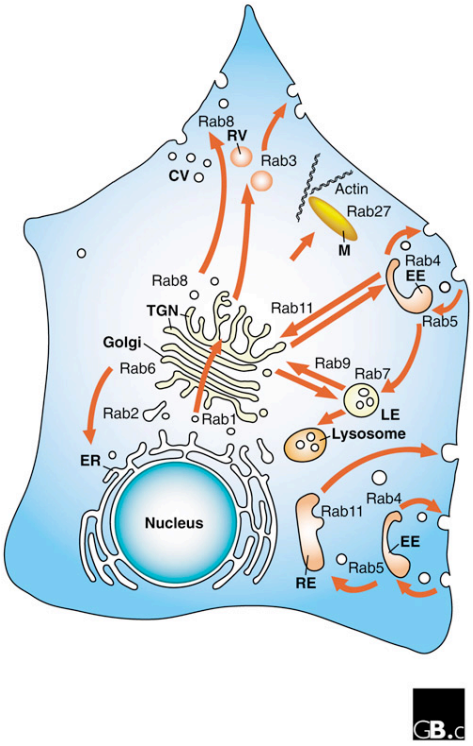


Figure 6: Scheme of a cell with different kinds of vesicle transport pathways. Red arrows indicate the direction of transport. Names of important proteins involved in the process are mentioned. Cell compartments are named in bold letters (Stenmark and Olkkonen, Genome Biology, 2001).

1.4.1.1 Endocytosis

Different kinds of endocytosis exist, but they all lead to the same result, the internalisation of extracellular macromolecules, plasma membrane lipids and plasma membrane proteins (Grant and Sato, 2006). This is important, because through this the cell can take up nutrients, regulate the expression of cell surface receptors, present antigens or maintain polarity (Mukherjee *et al.*, 1997). Endocytosis plays an essential role in cell signalling, both in generating and receiving signals. Endocytosis modulates the strength of extracellular signals, either by downregulating signalling by internalizing receptors in the receiving cells or by degrading ligand in the tissue to limit the amount available. Endocytosis may also bring activated receptors in close proximity to their downstream effectors, facilitating the propagation of a signalling cascade within the cell (Grant and Sato, 2006).

Endocytosis also facilitates the propagation of signals from cell to cell through a tissue.

To this day the best-known endocytosis pathway is the clathrin-coated pit pathway, both in worms (Fares and Grant, 2002) as well as in mammals (Brodsky *et al.*, 2001).

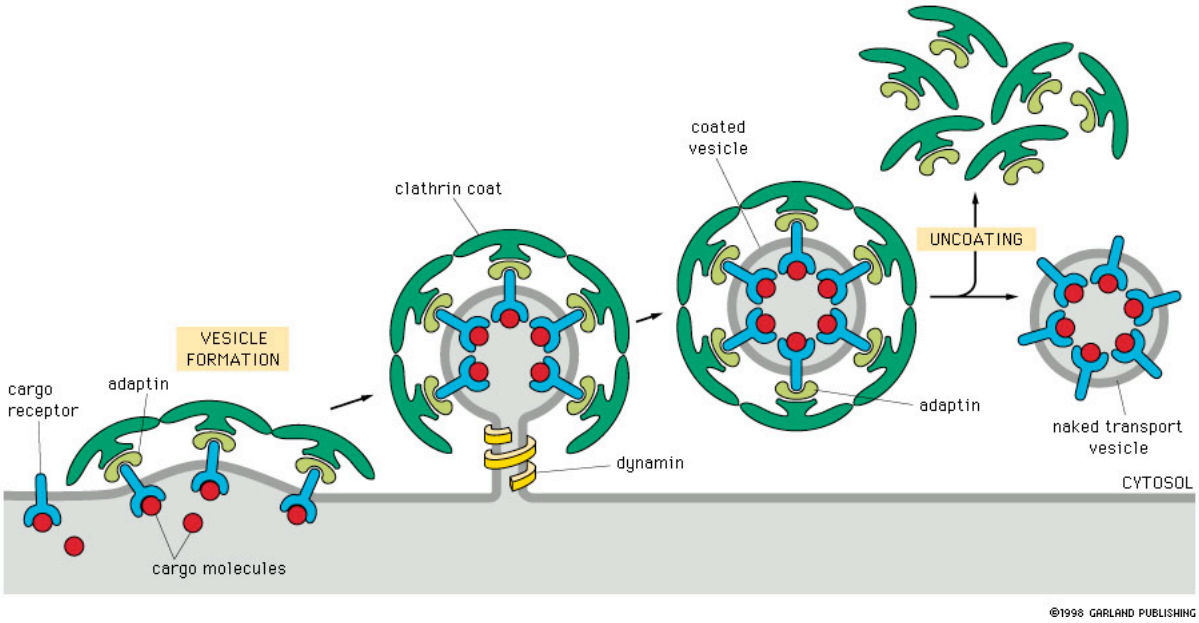


Figure 7: Formation of a clathrin-coated vesicle (Alberts *et al.*, 2002).

Receptors accumulate in the clathrin-coated pit, through association with clathrin. Adaptin serves as an adapter between clathrin and the receptor. Upon binding of the ligand, the vesicle forms and is later on pinched off the plasma membrane by the large GTPase dynamin (section 1.4.1.3). Afterwards the vesicles gets uncoated by the chaperone hsc70 and the DNA-J domain co-chaperone auxillin (Brodsky *et al.*, 2001). The receptors can then be recycled directly or in so-called recycling endosomes. Most ligands get degraded and are therefore transported from early to late endosomes and finally to lysosomes, where degradation takes place (Grant and Sato, 2006).

Clathrin-dependent endocytosis is used in *C. elegans* oocytes, where a vast number of yolk proteins and their associated lipids are endocytosed (Grant and Hirsh, 1999). The receptor for yolk is specifically expressed in oocytes and is similar to the human LDL-receptor (Grant and Hirsh, 1999). Mutations or RNAi-mediated depletion of *C. elegans* auxillin, adaptin, or clathrin all result in eggs that fail to take up yolk protein and die early in embryogenesis. The *C. elegans* Rab GTPases RAB-5, RAB-7 and RAB-11 are regulators of endocytosis, having homologs in all eukaryotes, and play a role in the uptake of yolk proteins (Grant and Hirsh, 1999).

In adult worms, coelomocytes have a high endocytotic activity. Coelomocytes are scavenger cells in the body cavity of *C. elegans* with a macrophage-like function (Fares and Greenwald, 2001). In addition endocytosis plays a role in signalling, where it can downregulate a signal by

internalizing the receptors. In other cases endocytosis may be required for signalling, by bringing the receptors in close proximity to its downstream effectors (Fares and Greenwald, 2001).

In one-cell *C. elegans* embryos, endocytic activity appears to be asymmetric following polarity establishment. Roughly three-fold more endocytic vesicles are found in the anterior half of the embryos compared to the posterior. The significance of this asymmetric distribution is unknown but it is under the control of the anterior-posterior polarity defined by the PAR proteins (Andrews and Ahringer, 2007).

1.4.1.2 Exocytosis

Exocytosis is the process by which cellular components, such as proteins and lipids are delivered to the plasma membrane or secreted into the extracellular space. Exocytosis essentially counteracts endocytosis and thus accomplishes many of the same functions. The newly generated proteins are first transported from the endoplasmatic reticulum to the Golgi apparatus in the early stages of exocytosis. They are transported through the four subcompartments of the Golgi, where some of the proteins get further modified. From there on they get transported to defined regions of the plasma membrane in different transport vesicles, where they are exocytosed. The proteins can also be delivered to the late endosomes or lysosomes (Lodish et al., 2007). Some surface receptors, which had been endocytosed together with the bound ligand, do not get degraded in lysosomes, but are transported back to the cell surface in so called recycling vesicles.

Mutations in proteins involved in exocytosis lead to severe or even lethal phenotypes (Grant and Hirsh, 1999; Roberts *et al.*, 2003), suggesting that exocytosis, like endocytosis, is essential for development. One example is SEC-23, a component of coat protein complex II-coated vesicles. These vesicles play a role in the transport from the endoplasmic reticulum to the Golgi apparatus. sec-23 mutants have effects like defective cuticle secretion and cannot undergo embryonic morphogenesis (Roberts *et al.*, 2003).

In adult worms, polarised secretion in polarised cells, like epithelial cells, provides an apical versus basolateral sorting mechanism, which allows proteins to be segregated to the correct membrane domain (Grant and Sato, 2006). Another example is the secretion of synaptic vesicles in neurons.

1.4.1.3 Dynamin

Dynamin is a large GTPase required to pinch off vesicles during both endocytosis and exocytosis. The dynamins are a big family (Collins, 1991), the first member having been isolated from

mammalian brain. The dynamin orthologue in *C. elegans* is called dyn-1, and it is very similar to its mammalian and *Drosophila* homologues. It is composed of a N-terminal GTPase, a pleckstrin homology domain, and a C-terminal proline-rich domain (Clark *et al.*, 1997). In *C. elegans*, it is needed for endocytosis, synaptic vesicle recycling, cytokinesis and the engulfment of apoptotic cell corpses. DYN-1 is localized to the mitotic spindle and, during subsequent stages of mitosis, it is concentrated at the metaphase plate, cytoplasmic vesicles, along spindle microtubules, and finally at the cleavage furrow and midbody (Thompson *et al.*, 2002).

A temperature sensitive mutant of *C. elegans* dyn-1 was isolated in 1997 by Clark et al.. At the permissive temperature dyn-1(ky51) worms are largely indistinguishable from wild type worms. After shift to the non-permissive temperature dyn-1 (ky51) worms show an uncoordinated phenotype within less than a minute. The mutation leads to sluggish movement, reduced pharyngeal pumping rate, prolonged defecation cycle and an egg-laying defect (Clark *et al.*, 1997).

1.4.2 Vesicle transport in polarity establishment and maintenance

It is already known that intracellular vesicle transport is needed during polarity establishment and maintenance in different kinds of cells in different organisms.

One of the most well studied examples of the involvement of vesicle trafficking during cell polarization is bud formation in the yeast *Saccharomyces cerevisiae*. The daughter cell (the "bud") grows from a single site on the mother cell. During this polarized growth, vesicle delivery is targeted to the bud site through trafficking on actin cables. The polarized reorganization of the actin cytoskeleton is driven by the small GTPase CDC-42, which is activated exclusively at the bud site. Vesicle trafficking to the bud site involves proteins like Sec4 and Rho3. The final steps in vesicle docking and secretion are mediated by the exocyst complex, consisting of Sec4, Exo70 and Exo84 (Zhang *et al.*, 2005), which activates SNAREs, enabling vesicle fusion with the plasma membrane (Whyte and Munro, 2002).

An example of the importance of vesicle trafficking in polarity establishment and maintenance in mammalian cells is Madin-Darby canine kidney (MDCK) epithelial cells. Vesicle trafficking, along with cell-cell adhesion, is necessary for development and maintenance of polarity. Cell-cell adhesion gives the initial cue for polarisation, then the cytoskeleton reorganises and specific membrane proteins are recruited via polarized delivery of transport vesicles containing basolateral membrane proteins, for instance. The proteins destined for either the apical or basolateral membrane are presorted in the trans-Golgi network, with the help of intrinsic sorting

signals and the clathrin-adaptor complex (Nelson, 2003). Without vesicle trafficking, the specialized membrane domains do not form and the cell remains functionally unpolarized.

The mechanisms for generating and maintaining cell polarity through the cytoskeleton and vesicle trafficking are conserved (Mostov et al., 2003). The tumour suppressor gene Lgl (lethal giant larvae) was first identified in *Drosophila melanogaster* (Gateff, 1978). It plays a role in epithelial polarity, specifically in creating two distinct membrane domains, the apical and basolateral domain (Bilder et al., 2000; Hutterer et al., 2004) and in asymmetric cell division of *Drosophila* neuroblasts. Specific cell fate determinants should be recruited to the basal cortex; this cannot be accomplished in the absence of Lgl (Ohshiro et al., 2000; Peng et al., 2000).

More recently, the budding yeast homologues, Sro7p and Sro77p (Zhang et al., 2005) of Lgl were identified. Sro7/77p interact with the exocyst complex and thereby positively regulate exocytosis. Sro7/77p are distributed all over the plasma membrane (Brennwald et al., 1994; Larsson et al., 1998; Lehman et al., 1999), while the exocyst complex is only located to the bud tip (Finger and Novick, 1998; Guo et al., 1999; TerBush and Novick, 1995). This is important for polarized cell growth. This may also reflect the mechanism by which Lgl contributes to cell polarization in *Drosophila*. The increased exocytosis facilitated by Lgl/Sro7/77p and SNARE function may contribute to cell polarisation (Zhang et al., 2005).

Vesicle transport also plays a role in polarization of the *Drosophila* oocyte. Oskar is essential for posterior patterning of the embryo and mediates this function through its localization to the posterior pole of the oocyte. Oskar protein stimulates endocytic recycling, which in turn contributes to the correct localization of Oskar (Vanzo et al., 2007).

So far there is not much known about the role of vesicle trafficking during polarity establishment and maintenance in *C. elegans*. Because vesicle transport plays an essential role in other polarized systems, the question arose if vesicle transport plays a role in polarity in *C. elegans*. More precisely, I was interested in the role of vesicle trafficking in the initiation of polarity establishment in one-cell *C. elegans* embryos.

2 Material and Methods

2.1 Primers

GFP constructs	Sequence
syn-4_f	ACTACTAGTCACCAAATCTC
syn-4_r	AGGCCTTTCCAACCCGGGA
exoc-7_f	ACTAGTATGTTGGTTGTTTA
exoc-7_r	AGGCCTGTTATTCTAGCAG
apt-9_f	ACTAGTATGGAGGAAGAA
apt-9_r	AGGCCTAAAATTAATATTAAAAATATG
sec-5_f	ACTAGTATGGAAGAAAACGC
sec-5_r	AGGCCTGATGTTGGAGGTGT
spd-2_f	ACTAGTATGGAAGATGATGC
spd-2_r	AGGCCTCTTTCTATTCGAAA
spd-5_f	ACTAGTATGGAGGATAATTCTGTGC
spd-5_r	AGGCCTCTTTTTGCGATTTCC
exoc-7neu_f	ACTAGTATGTTGTTTTTATTATTTTTATTGTTTTCC
exoc-7neu_r	AGGCCTGTTAATTCTAGCAGATGAGAATAGATTTCG
cogc-2_f	ACTAGTATGGGTACACTTCATGGCG
cogc-2_r	AGGCCTAGCATTCCCCGCTTCTT
Y47G6A.18_f	ACTAGTATGTCGGACGGTCAGC
Y47G6A.18_r	AGGCTCTTGCTGAACGCCTC
RNAi feeding constructs	
syn-4RNAi_f	CGTCATGTCTTGACTACGCTG
syn-4RNAi_r	GCCTTGGCTTTGTACCAATA
Sequencing primers	**Sequence**
exoc-7_f-seq1	CGCAGAAGCACAGAAATCATC
exoc-7_f-seq2	GATCCACAGCAGTCCGCAA
exoc-7_f-seq3	GTCGATTTTGATTATCAGAA
exoc-7_f-seq4	CGACAACTGCCTCTGTAAT
exoc-7_f-seq5	CGATGATCCAACACTTGCAA
Test for GFP construct	**Sequence**
gfp-rab-5_f	CAGGTGCTGAAGTCAAGTTTGA
gfp-rab-5_r	GTCGACTCCTTGAATTCATCCC

Table 1: Sequences of primers for GFP-constructs, RNAi feeding constructs, sequencing and control primers for GFP-constructs.

2.2 Vectors

2.2.1 N-GFP:

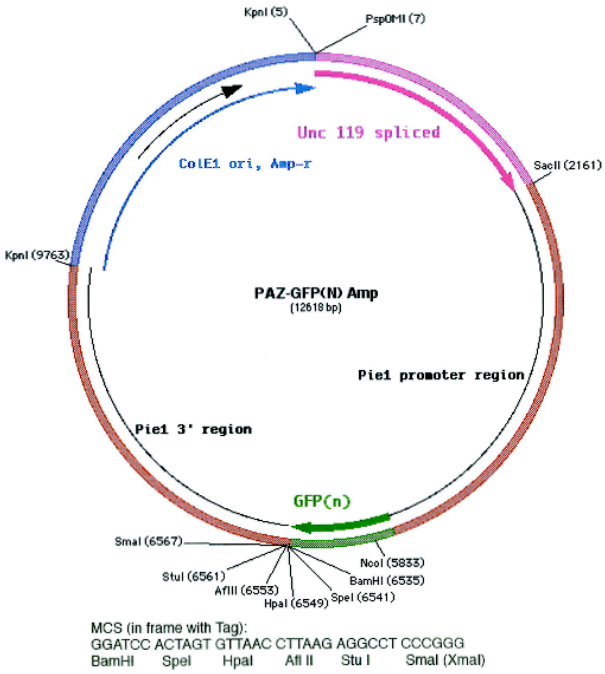


Figure 8: schema of the PAZ-GFP(N) Amp vector; Stu I and Spe I restriction sites were used for insertion of the gene (Praitis *et al.*, 2001).

2.2.2 C-GFP:

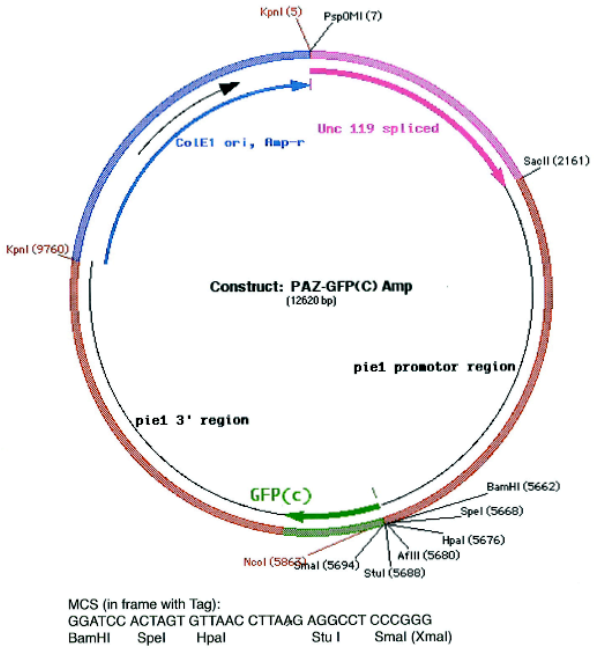


Figure 9: schema of the PAZ-GFP(C) Amp vector; Stu I and Spe I restriction sites were used for insertion of the gene (gift of Andrei Pozniakowsky, MPI-CBG, Dresden).

2.2.3 N-mCherry:

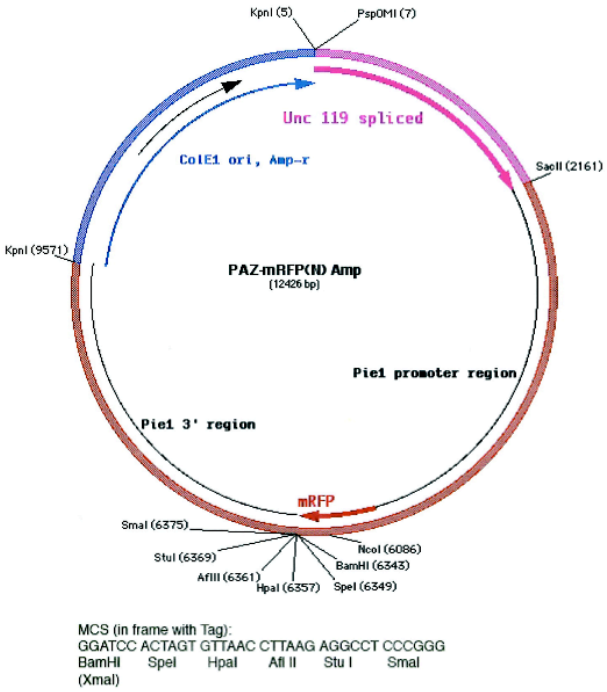


Figure 10: schema of the PAZ-mRPF(N) Amp vector and the sequence of the multiple cloning site; Stu I and Spe I restriction sites were used for insertion of the gene (gift of Andrei Pozniakowsky, MPI-CBG, Dresden).

2.2.4 L4440:

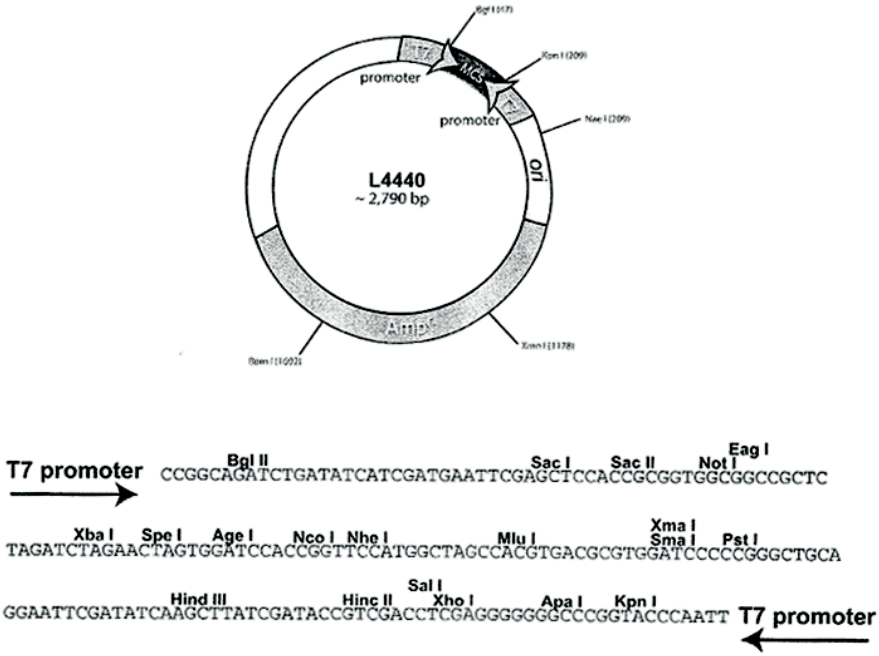


Figure 11: schema of the L4440 vector with the sequence of the multiple cloning site; Sma I restriction site was used for insertion of the gene (Timmons *et al.*, 2001).

2.3 Enzymes

All enzymes that I used in my project were produced by Fermentas.

Enzyme	Name	#	Buffer	#
polymerases	High Fidelity PCR enzyme mix	K0191	10x High Fidelity PCR Buffer +MgCl2	K0192
	Taq DNA Polymerase LC	EP0404	Taq buf. +KCl - MgCl2	
			25 mM MgCl2	
			10 dNTP mix	R0192
ligase	T4 DNA Ligase	EL0011	Buffer T4 DNA Ligase	
restriction enzymes	Eco174l (StuI)	ER0421	Tango buffer	BY5
	BcuI (SpeI)	ER1251		
	SmaI	ER0661		
phosphatase	Calf Intestinal Alcaline Phosphatase	EF0341		

Table 2: Enzymes and appropriate buffers

2.4 Worm strains

Strain	Genotype	In text referred as
N2		N2
CX51	dyn-1(ky51)	CX51 dyn-1(ky51ts)
RT122	RAB-5::GFP(pwIs20);unc-119(ed3)	RAB-5::GFP
	RAB-5::GFP[5'pie-1::GFP::rab-5(genomic)::3'pie-1::unc-119(+)]	
JJ1473	NMY-2::GFP(zuIs45);unc-119(ed3)	NMY-2::GFP
	NMY-2::GFP[5'nmy-2::NMY-2(genomic)::GFP::3'nmy-2::unc-119(+)]	
TH25	C47B2.3::GFP(ddIs8);unc-119(ed3)	C47B2.3::GFP
	C47B2.3::GFP[5'pie-1::C47B2.3(genomic)::GFP::3'pie-1::uncß119(+)]	
UE5	EXOC-7::GFP(oaIs1);unc-119(ed3)	EXOC-7::GFP
	EXOC-7::GFP[5'pie-1::exoc-7(genomic)::GFP::3'pie-1::unc-119(+)]	
KK0911	PAR-3::GFP;unc-119(ed3)	PAR-3::GFP
	PAR-3::GFP[5'pie-1::par-3(genomic)::GFP::3'pie-1::unc-119(+)]	
WH0327	SP12::GFP(ojls23);unc-119(ed3)	SP12::GFP
	SP12::GFP[5'pie-1::sp12(genomic)::GFP::3'pie-1::unc-119(+)]	
DP38	unc-119(ed3)	DP38
UE6	dyn-1(ky51);RAB-5::GFP(pwIs20)	dyn-1(ky51ts);RAB-5::GFP
UE7	dyn-1(ky51);C47B2.3::GFP(ddIs8)	dyn-1(ky51ts);αtubulin::YFP
UE8	dyn-1(ky51);NMY-2::GFP(zuIs45)	dyn-1(ky51ts);NMY-2::GFP

UE9	dyn-1(ky51); EXOC-7::GFP(oaIs1)	dyn-1(ky51ts);EXOC-7::GFP
UE10	dyn-1(ky51); PAR-3::GFP(#)	dyn-1(ky51ts);PAR-3::GFP

Table 3: Worm strains that were used during this project.

2.5 Worm maintenance

Worms were kept on NGM/OP50 plates at 16°C. 3-5 hermaphrodites were passed to new plates every 3-4 days.

2.6 Cloning

2.6.1 PCR

Basic PCR mix (50µl reaction)

primer 1 (100pmol/µl)	0.25µl
primer 2 (100pmol/µl)	0.25µl
Template DNA	1µl
10x PCR buffer	5µl
dNTP mix (10mM)	0.5µl
Polymerase	1µl
Water	42µl

Table 4: PCR mix for amplifying the gene for GFP-constructs; I used genomic N2 DNA as template DNA and the High Fidelity PCR enzyme mix as polymerase.

The polymerase used was the High Fidelity Enzyme Mix and the adequate buffer, the 10xHigh Fidelity PCR buffer +$MgCl_2$. For the PCR I used the BioRad DNA Engine, Peltier Thermal Cycler.

PCR program (genomic PCR)

95°C	5:00 min
25 cycles 95°C 50-54°C 68°C	 0:45 min 0:45 min 1:00 min/kb target
68°C	5:00
4°C	for ever

Table 5: Basic program for the PCR reaction; the time of the elongation step was adjusted according to the size of the gene and the annealing temperature of the annealing step was chosen between 50 and 54°C according to the individual primers.

The PCR was analysed on a 1% agarose gel, with 1:10000 ethidium bromide added.

3µl Gene Ruler Express DNA ladder marker and 5µl sample (+1µl 6x Orange Loading Dye) were loaded on the gel. The gel was run at 125mA for 15min.

2.6.2 PCR Purification

For purification of PCR products, I used the QIAGEN PCR purification kit. The protocol was used according to the manufacturers protocol. The PCR product was eluted in 50µl water.

2.6.3 Restriction digest

PCR digest (25µl)		**Vector digest (**15µl)	
10x Tango buffer	2,5µl	10x Tango buffer	1,5µl
SpeI (BcuI)	1,25µl	SpeI (BcuI)	0,75µl
StuI (Eco147I)	1,25µl	StuI (Eco147I)	0,75µl
PCR product	20µl	vector	5µl
		water	7µl

Table 6: Basic mixes for restriction digests of PCR products and vectors

I incubated restriction digests for 2 hours or over night at 37°C.

2.6.4 Gel purification

I usted the QIAquick Gel Extraction Kit Protocol according to the manufacturers protocol. The PCR products were eluted in 50µl water, the vectors in 30µl.

2.6.5 Ligation

Ligation	
PCR product	7µl
Vector	1µl
10x T4 ligase buffer	1µl
T4 ligase	1µl

Table 7: 10µl ligation reaction

The ligation was incubated over night at 16°C.

2.6.6 Transformation (Elektroporation)

For transformation of the vector (+instert) I used electrocompetent DH5α bacteria, which were stored at -80°C. 50µl of the bacteria were thawed on ice and 1µl of the ligation product was added. The mixture was transferred to pre-chilled electroporation cuvettes. The bacteria were electroporated at 2,5 amp, with the BioRad *E. coli* Pulser. After the elecroporation 1ml of LB was added immediately to the bacteria. The bacteria were allowed to recover for 30min at room temperature. Afterwards the bacteria were plated on LB +amp plates and incubated on 37°C over night.

On the next day the colonies were checked by colony PCR and positives were sequenced to see if the sequence

2.7 Bombardment

2.7.1 Growing the worms

The plates (9 cm) were made with peptone medium [per liter: 1,2 g NaCl; 20g bactopeptone; 25g agar; after autoclaving: 1ml cholesterol (5mg/ml); 1ml 1M MgSO4; 25ml 1M KH2PO4, pH 6]. E. coli were grown in LB over night and then seeded on the peptone plates (1ml/plate). They grew for 1-2 days, before the worms were seeded onto the plates. DP38 [unc-119(ed3)], that were maintained on an NGM/OP50 plates were transferred to the peptone. 5-10 worms were used for each plate. The worms could grow on the plates at 25°C for about 10 days until all the bacteria were gone.

2.7.2 Preparing the DNA gold beads

BioRad 1,0 µ gold beads (cat no. 1652263) were used for the procedure. Approximately 0,6 mg gold beads were used per bombardment. For 10 bombardments 6 mg beads were used. 1ml of 70% ethanol was added and after vortexing and spinning the supernatant was removed. Afterwards the beads were washed three times with water and finally resuspended in10µl 50% glycerol per bombardment. Now the beads were ready for absorption of DNA. Beads were vortexed for about 5 min and per bombardment 1µl DNA, 10µl 2,5M CaCl and 4µl 0,1M spermidine were added with 1min vortex steps in-between. After vortexing again the supernatant was removed and the beads were washed three times with 70% ethanol. In the end the beads were resuspended in 10µl 100% ethanol per bombardment and vortexed again.

2.7.3 Bombarding the worms

The BioRad PDS-1000/He system (cat no. 1652257) was used for bombardment of the worms. The beads were immobilised on washed (in 100% ethanol) and dried macrocarriers (BioRad macrocarriers, cat no. 1652322) lying in macrocarrier holders (BioRad macrocarrier holders, cat no. 1652336). Therefore 10µl of the bead suspension was aliquoted onto the center of each macrocarrier and dried at room temperature.

The worms were washed off the 9 cm fully-eaten peptone/C600 plates with 0,1M NaCl and collected into a 50 ml falcon tube. The supernatant was removed, leaving approximately 100µl per bombardment. The worms were resuspended and the suspension was pipetted onto the center of the OP50 lawn of a 4 cm NGM plate. One plate per bombardment was used. It took 5-10 min until the excess liquid was soaked into the plate.

For the bombardment the BioRad rupture disc (cat no. 1652330) was placed into the rupture disc holder, a BioRad stopping screen (cat no. 1652336) was placed into the bottom of the macrocarrier holder assembly and the worms were placed in the center of the specimen platform. A vacuum of about 28 inches was applied before firing (rupture disc had to break). Afterwards the chamber was vented immediately.

2.7.4 Post-bombardment worm care

The worms were allowed to recover over night on the bombarded plate. They were washed off with 0,1M NaCl on the next day and split on 2-3 9 cm NGM/OP50 plates.

The worms were screened after 7-10 days on 25°C. The transformants were checked for GFP by fluorescence microscopy.

2.8 Crosses

For the crosses I always used 10 males of the CX51 dyn-1(ky51ts) strain and 5 hermaphrodites of the GFP strain (Figure 12).

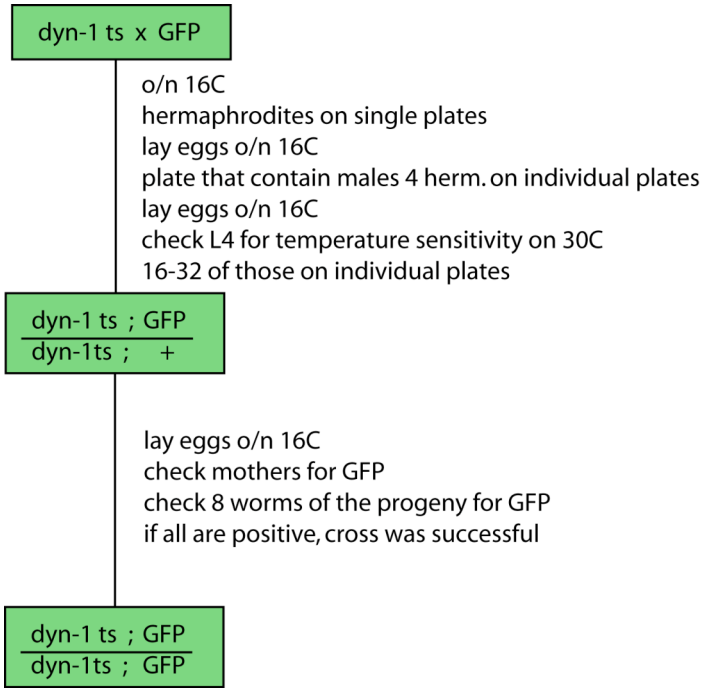


Figure 12: Outline of the cross between two worm strains. The dyn-1 temperature sensitive strain was crossed to a GFP strain to generate a dyn-1 temperature sensitive line containing a GFP tagged protein.

The worms were checked for GFP with a fluorescent microscope.

2.8.1 Cross to obtain dyn-1(ky51ts) males

Before the crosses could be performed, dyn-1(ky51ts) males had to be generated. A cross was performed with N2 males and CX51 dyn-1(ky51ts) hermaphrodites. The males in the progeny of the first mating were crossed again to CX51 dyn-1(ky51ts) hermaphrodites and the males in the progeny were tested for temperature sensitivity, meaning worms were put on 30°C for 30 min and later checked for the temperature sensitive phenotype. The males were maintained by adding CX51 dyn-1(ky51ts) hermaphrodites. From that time point on they were ready to be used for the other crosses.

2.8.2 Heat shock to obtain dyn-1(ky51ts) males

Heat shock was the second method to get CX51 dyn-1(ky51ts) males. About 20 L4 larvae or young adults were transferred to a NGM/OP50 plate and incubated on 30°C for 5 hours. Afterwards they could grow on 16°C for about 3 days.

The first method using the cross was more effective than the heat shock.

2.9 RNAi

2.9.1 Making RNAi feeding bacteria

First the L4440 vector had to be T-tailed. Prior to this it had to be digested and cleaned with the QIAGEN PCR purification kit (as explained above).

blunt-end digest

L4440	10µl
Water	6µl
10x Tango buffer	2µl
SmaI	2µl

Table 8: Blunt-end digest, 20µl reaction; for the T-tailing of the L4440 vector.

The digest was incubated on 37°C for two hours or longer.

t-tailing reaction (50µl reaction)

DNA	25µl
Water	12µl
10x PCR buffer	5µl
25 mM MgCl2	5µl
100 mM dTTP	2µl
Taq	1µl

Table 9: Tailing reaction for L4440 vector; 50µl reaction.

The tailing reaction took two hours at 72°C. Thereafter it was cleaned with the QIAGEN PCR purification kit (as explained above) and eluted in 30µl water.

The insert was amplified by PCR and also cleaned with the QUIAGEN PCR purification kit.

PCR (amplify insert)

10x PCR buffer	5µl
25 mM MgCl2	5µl
primer 1	0.25µl
primer 2	0.25µl
dNTP mix	0.25µl
N2 genomic DNA	0.5µl
taq	0.5µl
water	38.25µl

Table 10: PCR reaction for the amplification of the insert for making a RNAi feeding construct. Taq polymerase was used as enzyme and N2 genomic DNA as template.

The next step was the ligation of the PCR product with the vector and the transformation into HT115 bacteria.

Ligation

insert (PCR product)	8µl
t-tailed L4440	1µl
10x T4 ligase buffer	1µl
T4 ligase	1µl

Table 11: Ligation reaction for the L4440 vector and the insert for the RNAi feeding construct.

For transformation 1,5µl of the ligation was added to 50µl of HT115 bacteria and the mixture was transferred to pre-chilled electroporation cuvettes. After the shock at maximum amperage 1ml of LB medium was added immediately. The bacteria were allowed to recover for 30min at room temperature. Afterwards the bacteria were plated on LB +amp plates and incubated on 37°C over night.

On the next day the colonies could be checked for positive clones, by colony PCR. Each single colony was transferred into 5µl of water and this mixture was used for colony PCR.

Colony PCR

10x PCR buffer	2.5µl
25 mM MgCl2	5µl
T7 primer	0.5µl
dNTP mix	0.25µl
DMSO	1µl
Taq	0.25µl
Water	12.5µl

Table 12: Colony PCR for testing the colonies for positive clones; Taq polymerase was used as enzyme and 1µl of colonies diluted in 5µl water were used as template.

10µl of the PCR products were run on a 1% agarose gel to check for positive clones. Bacteria containing correct clones were grown to saturation then stored in 40% glycerol at -80°C.

2.9.2 RNAi by feeding

RNAi feeding bacteria were inoculated in 2ml LB +amp+tet at 37°C over night. On the next day 100µl of the liquid culture were diluted in 5ml LB +amp and incubated for 3 hours at 37°C. 15µl IPTG (1M) were added and the tube was incubated again for 30min at 37°C.

Finally the bacteria were seeded onto NGM plates (with IPTG), 0,25ml on each 4cm plate and kept at room temperature over night to dry. Afterwards the plates were stored at 4°C.

For the experiment the worms were put onto the RNAi plates 2 to 48 hours before recording depending on the construct used.

2.10 Microscope

2.10.1 Time-lapse microscopy

Worms were dissected in 2.5 µl 0.1 M NaCl+4% sucrose solution on an 18x18 mm coverslip to release early embryos. Coverslips with embryos were inverted onto a 2% agarose (0.1 M NaCl + 4% sucrose) pad on a standard microscope slide for viewing. DIC images and wide-field epifluorescence images of Rab-5::GFP, α tubulin::YFP, NMY-2::GFP were recorded with a 40X Plan NeoFluor 1.3 NA lens (Zeiss) on a wide wield epifluorescence microscope (Zeiss Axiovert 200M) equipped with a cooled CCD camera (CoolSnap HQ, Photometrics) and eGFP filter set (F46-002, Chroma). An external shutter (Uniblitz) was used to control illumination. Spinning disk imaging of GFP-lines (Rab-5, EXOC-7, SP12) was performed with a 100X Plan FLUAR 1.45 NA lens (Zeiss) using a Nipkow spinning disk head (VisiTech International Ltd.) and a cooled CCD camera (CoolSnap HQ, Photometrics) at 2x binning. Illumination was supplied by a fiber-optic-coupled 488 nm solid state laser (Coherent Inc.) and controlled through an external shutter (Uniblitz) on the spinning disk. The stage was controlled by a motorized XYZ driver (Applied Scientific Instruments). All components and image acquisitions were controlled by MetaMorph (7.0r4) software.

2.10.2 Temperature switch

The intervals between the single frames were 5-10 seconds long. For the recordings with the temperature sensitive mutant two 40x lenses were used. One was at room temperature (25-28°C) and the other one was heated up to 31°C. The two lenses were situated next to each other, so the temperature of the sample could be switched during time-lapse recording.

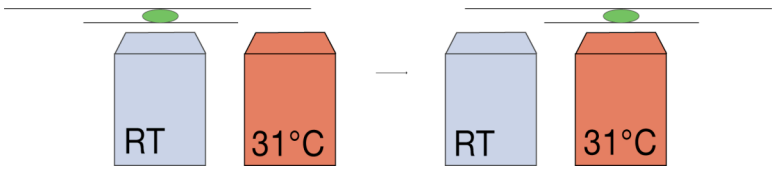


Figure 13: diagram of lens switching; the two lenses are next to each ohter, one is at RT (25-28°C), the other one is at 31°C; embryos can be shifted to the higher temperature at a certain timepoint during timelapse microscopy.

3 Results

The main question of my project was how vesicle transport influences polarity establishment in *C. elegans* one-cell embryos.

Therefore I chose the protein dynamin as a good candidate to look at, as it is involved in clathrin-coated vesicle transport. An advantage in this case was that a temperature sensitive mutant of the dyn-1 gene already existed, which also had been published (Clark et al., 1997). It was necessary to have a temperature sensitive mutant of dynamin because with this it was possible to turn off the protein only at a specific time point, like polarity establishment. Dynamin has so many functions in the cell and therefore loss of function mutants are lethal, the RNAi in adults causes sterility in the F0 generation and partial RNAi is difficult to interpret. I tried the partial dyn-1 RNAi before I started working with the temperature sensitive mutant but it turned out that the experiments could not be performed with this technique.

3.1 dyn-1(ky51ts)

Before I started the experiments it was important to prove that the dyn-1(ky51ts) mutant behaves like the wild type at room temperature. In the experiments with the temperature sensitive mutant I recorded with two different 40x lenses, one at room temperature (25-28°C) and one at hot lens at 31°C. Depending on the experiments I switched the lenses (from room temperature to 31°C) before, during or after polarity establishment in the one-cell embryo.

The mutant behaves like the wild type at room temperature. Polarity establishment and cytokinesis can be completed normally (Figure 14). In this experiment and throughout all the other experiments I judged polarity establishment by smoothing of the cortex on the posterior side of the embryo while the other side is still ruffling. Before polarity establishment the cortex around the whole embryo has surface contractions.

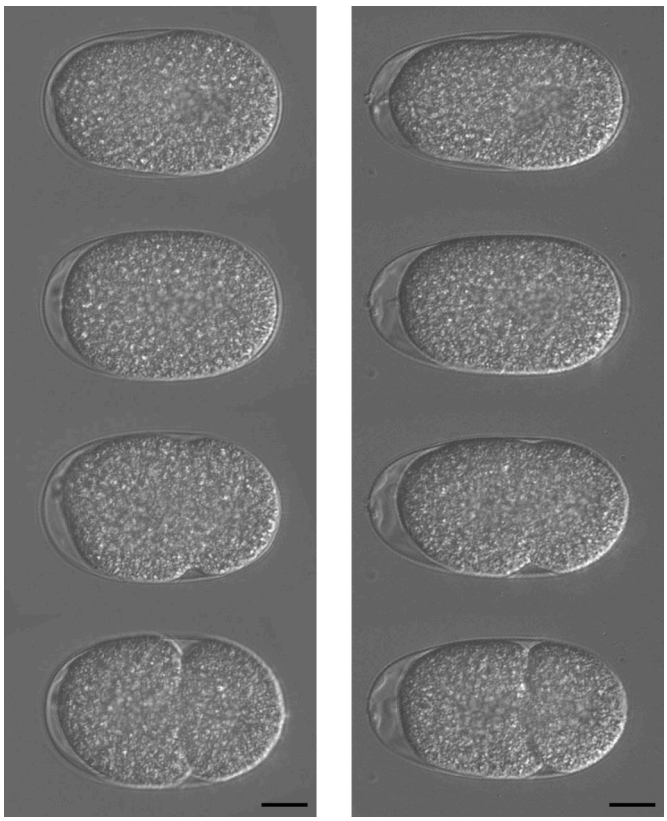


Figure 14: time lapse recordings of N2 (wild type; left panel) and CX51 dyn-1(ky51ts) (right panel) at room temperature. Pictures were chosen according to the cell cycle stages. Bar, 10µm. (N2: n=3; CX51 dyn-1(ky51ts): n=6).

3.2 Dynamin is required for cytokinesis

It has already been published, that the one-cell embryo cannot undergo cytokinesis in the absence of dynamin (Thompson et al., 2002). With the temperature sensitive dyn-1 mutant I wanted to check for this phenotype.

The dynamin mutant was observed at two different temperatures, at a lower temperature, where DYN-1 was still functional and at a restrictive temperature, which resulted in the loss of function of DYN-1.

I made time-lapse phase contrast images with the dyn-1(ky51ts) mutant first at room temperature from the end of polarity establishment until pronuclear meeting. At the time the two pronuclei met, I switched the lens and continued recording with the 31°C lens until cytokinesis (Figure 15). The same procedure was performed with N2 (wild type) worms as a control. The second control was the CX51 dyn-1(ky51ts) mutant only at room temperature without, temperature shift, where DYN-1 is still functional (Figure 15; right panel).

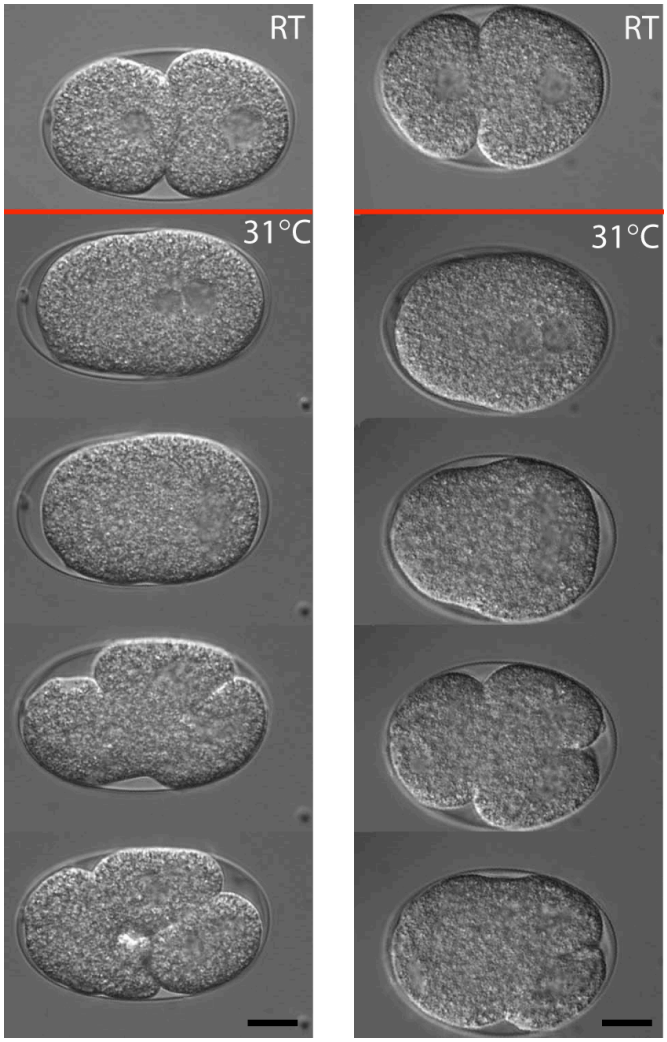


Figure 15: time lapse recordings of one-cell embryos at five different time points during first cell division; wild type (left), CX51 dyn-1(ky51ts) mutant (right). First at RT, the red line indicates the shift to 31°C, followed by pictures at 31°C at four different stages. The time points were taken in relative to the shift. Bar, 10 µm. (N2: n=2; CX51 dyn-1(ky51ts): n=3).

In the wild type the cell could still divide into two separated cells, while the mutant could not. In the dyn-1(ky51ts) mutant an initial ingression of the cleavage furrow could be seen, but this furrow regressed again after some time (Figure 15).

This experiment shows the expected result, confirming that the temperature shift regimen of my experimental set-up was effective in inactivating dynamin. As mentioned before, it had already been published that dynamin function is required for cytokinesis (Thompson et al., 2002).

3.3 Does dyn-1 affect NMY-2 distribution?

NMY-2, non-muscle myosin II, is maternally expressed and needed for several events happening during asymmetric cell division. Among them are correct cytoplasmic flow and PAR protein distribution, cytokinesis and proper anterior-posterior polarity (Guo and Kemphues, 1996). I was interested if the loss of DYN-1 function has an effect on NMY-2 distribution.

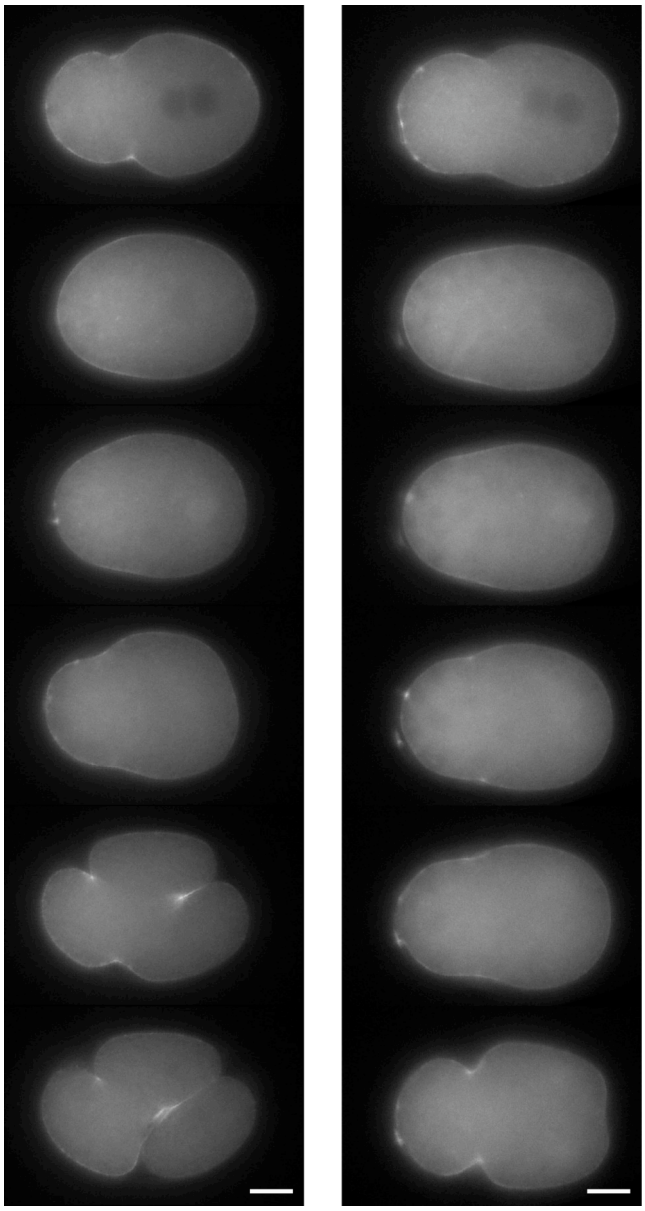


Figure 16: time lapse recordings of NMY-2::GFP (488nm). The images were taken in an interval of 2 minutes. NMY-2::GFP is shown the left panel the mutant dyn-1(ky51ts);NMY-2::GFP in the right one. The shift to 31°C was made upon pronuclear meeting. The first pictures in both panels show the embryo right after the shift. Bars,10µm. (NMY-2::GFP: n=1 RT, n=4 31°C; dyn-1(ky51ts);NMY-2::GFP: n=1 RT, n=3 31°C)

I used the dynamin temperature sensitive strain that contained NMY-2::GFP to see the effect of deactivation of dynamin on NMY-2.

For the experiment the NMY-2::GFP and the CX51 dyn-1(ky51ts) strain had to be crossed to give a new strain, dyn-1(ky51ts);NMY-2::GFP.Time lapse fluorescent images were taken from dyn-1(ky51ts);NMY-2::GFP embryos at room temperature and the restrictive temperature for the temperature sensitive embryo. NMY-2::GFP strain served as control at both temperatures. The temperature was shifted from room temperature to 31°C at pronuclear meeting and the embryos were imaged until cell division. This allowed me to look at a later stage of asymmetric cell division.

In the wild type, NMY-2 distributed asymmetrically to the anterior side of the embryo. Also at 31°C the NMY-2::GFP embryo, which serves as a control, showed this effect. The mutant, dyn-1(ky51ts);NMY-2::GFP showed a very similar distribution pattern of NMY-2 at both room temperature and 31°. No significant differences in NMY-2 distribution could be determined between the control and the mutant. In all cases NMY-2 was accumulated at the site of the furrow ingression. At 31°C there was a high concentration of protein at the leading edge of the cytokinesis furrow in the wild type embryo. In the mutant this could not be detected so clearly but this can be due to the fact that there was only little ingression of the furrow (Figure 16).

This brings me to the conclusion that dynamin function has no effect on NMY-2 distribution during the later stages of the cell cycle. Myosin is still present at the cortex.

3.4 Spindle positioning

The spindle gets dispositioned in embryos that were recorded at 31°C. So the question arose, if this is the cause of the cytokinesis defect in the dynamin temperature sensitive mutants. Is incorrect spindle positioning dependent on the dyn-1 mutation?

3.4.1 Phase contrast microscopy

I re-examined the time-lapse images from my initial experiments inactivating dynamin. The phase contrast images (shown in Figure 15) showed a mispositioned spindle in the dyn-1(ky51ts) mutant at 31°, but also in the wild type at higher temperatures.

The problems in spindle positioning may be due to the high temperature and not due to the temperature sensitive mutation in dyn-1, because the defect is visible in wild type and mutant embryos.

3.4.2 α tubulin::GFP fluorescence

The problem in spindle positioning can already be seen in the DIC recordings, but with α-tubulin::YFP time lapse fluorescent images I wanted to get a clearer view at the situation in the embryo.

I analyzed spindle positioning using fluorescent α-tubulin in dyn-1(ky51ts) mutants during cell division at the restrictive temperature.

Therefore a cross to create dyn-1(ky51ts);α-tubulin::YFP was performed. One-cell dyn-1(ky51ts);α-tubulin::YFP embryos were used for time lapse fluorescent imaging at room temperature and 31°C. Recordings started pronuclear meeting and the temperature was shifted immediately to 31°. The α-tubulin::YFP control was treated the same way and both strains were also recorded at room temperature without any shift to serve as controls.

In both control α-tubulin::YFP embryos and dyn-1(ky51ts); α-tubulin::YFP embryos, the spindle positioning was incorrect. The spindle was turned up to an ankle of 90° compared to the wild type. The pictures (Figure 17) were taken at the same time points relative to the shift to 31°C, but it was quite obvious that mitosis took longer in the dyn-1(ky51ts); α-tubulin::YFP mutant than in the wild type.

This indicates that the displaced spindle positioning is not due to the inactivation of dynamin, but rather due to the high temperature, at which the dynamics of many components of the embryo get out of control, for example the microtubule growth and shrinkage. This phenotype is similar to the one in mutations in microtubule stabilising factors (zyg-9) (Matthews et al., 1998). Nevertheless it is again clearly visible that the wild type can still undergo cytokinesis, while the temperature sensitive mutant cannot, so the mispositioning of the spindle is not causative of the cytokinesis effect.

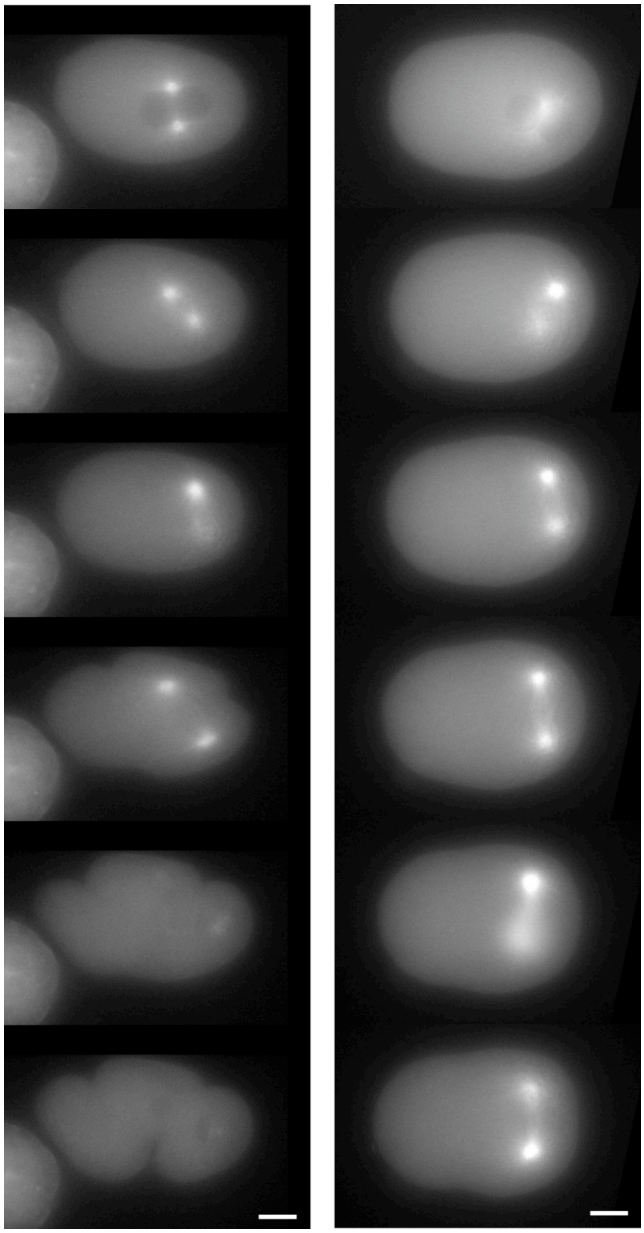


Figure 17: time lapse recordings at 31°C with 1 min intervals. On the left side α tubulin::YFP and on the right side dyn-1(ky51ts); α-tubulin::YFP. Bars, 10µm. (α tubulin::YFP: n=3 RT, n=4 31°C; dyn-1(ky51ts);α tubulin::YFP: n=3 RT, n=2/3 31°C)

3.5 Does dyn-1 affect vesicle dynamics?

The next aim was to look at a component of the vesicle transport to determine the effect of inactivation of dyn-1. The candidate we chose was RAB-5, which is located at the early endosomes. Endocytosis is known to require dynamin.

I observed the early endosomes at room temperature and at the restrictive temperature in dyn-1(ky51ts) mutant embryos.

To make this possible I crossed the CX51 dyn-1(ky51ts) strain with a strain that contained RAB-5::GFP. This new dyn-1(ky51ts);RAB-5::GFP line was used to make time lapse recordings with the fluorescent microscope. Again the imaging started at the end of polarity establishment until pronuclear meeting at room temperature and continued at 31°C from pronuclear meeting until cytokinesis. Wild type RAB-5::GFP embryos were also recorded at 31°, serving as one control, and both strains, dyn-1(ky51ts);RAB-5::GFP and RAB-5::GFP recorded at room temperature without shift to the restrictive temperature provided additional controls.

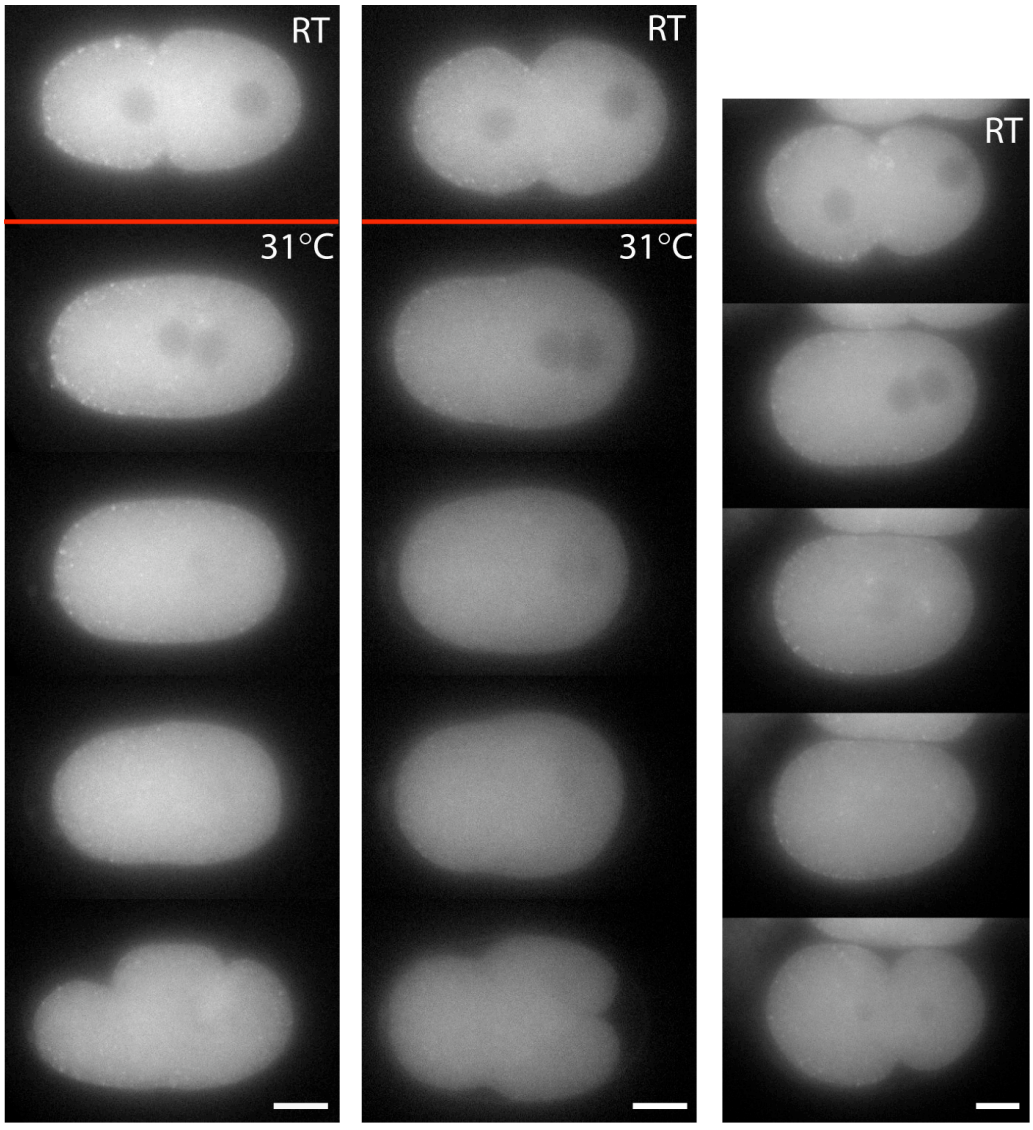


Figure 18: time lapse recordings (as in figure 9); RAB-5::GFP (left panel), dyn-1(ky51ts);RAB-5::GFP (middle panel) and dyn-1(ky51ts);RAB-5::GFP without temperature shift (right panel). Images were taken at first at room temperature, the red line indicates the shift to 31°C, followed by pictures at 31°C at four different stages during division from one to two-cell stage embryos. The timepoints were taken relative to the shift. Bar, 10 µm. (RAB-5::GFP: n=3 RT, n=4 31°C; dyn-1(ky51ts);RAB-5::GFP: n=1 RT, n=3 31°C).

In wild type embryos (RAB-5::GFP) (Figure 18, left panel), the endosomes were clearly visible as small GFP dots concentrated near the anterior cortex. Even after the shift to 31°C until cytokinesis the dots were still present at the cortex. In dyn-1(ky51ts);RAB-5::GFP mutants (Figure 18, middle panel), it seemed to be different. Shortly after the shift to the restrictive temperature the signal of the endosomes became significantly weaker. At cytokinesis, nearly no vesicles seemed to be present any more. In contrast, time lapse images of the dyn-1(ky51ts);RAB-5::GFP mutant at room temperature showed the signal of the endosomes throughout the whole experiment.

The results from this experiment may mean that the function of dynamin is important for vesicle dynamics. This seems to be consistent with the fact that dynamin cleaves off the vesicles from the plasma membrane during endocytosis (McNiven et al., 2000).

3.6 Is dynamin required for polarity establishment?

3.6.1 Time lapse recordings

At this point I wanted to answer the question if dynamin is required for polarity establishment and, if so, at what time during the process is it needed.

3.6.1.1 Initiation of polarity establishment

I looked at dyn-1(ky51ts) mutant and wild type embryos that were shifted to 31°C before polarity establishment to analyse the formation of the posterior domain.

For these experiments early embryos from N2 (wild type) and CX51 dyn-1(ky51ts) worms were chosen, which were still ruffling and were just about to initiate polarity establishment. Before polarity initiation, they were shifted to 31°C and the process was documented by time-lapse fluorescent imaging. The first picture in Figure 19 shows the embryos right after the shift to the higher temperature and later pictures in an interval of 2 minutes were picked.

It showed that in comparison to the wild type, the CX51 dyn-1(ky51ts) mutant could not establish polarity when shifted to the restrictive temperature before the initiation of polarity establishment. It still had an uneven shape without any smooth domain. The wild type, on the other hand, had already started to develop the posterior domain (Figure 19).

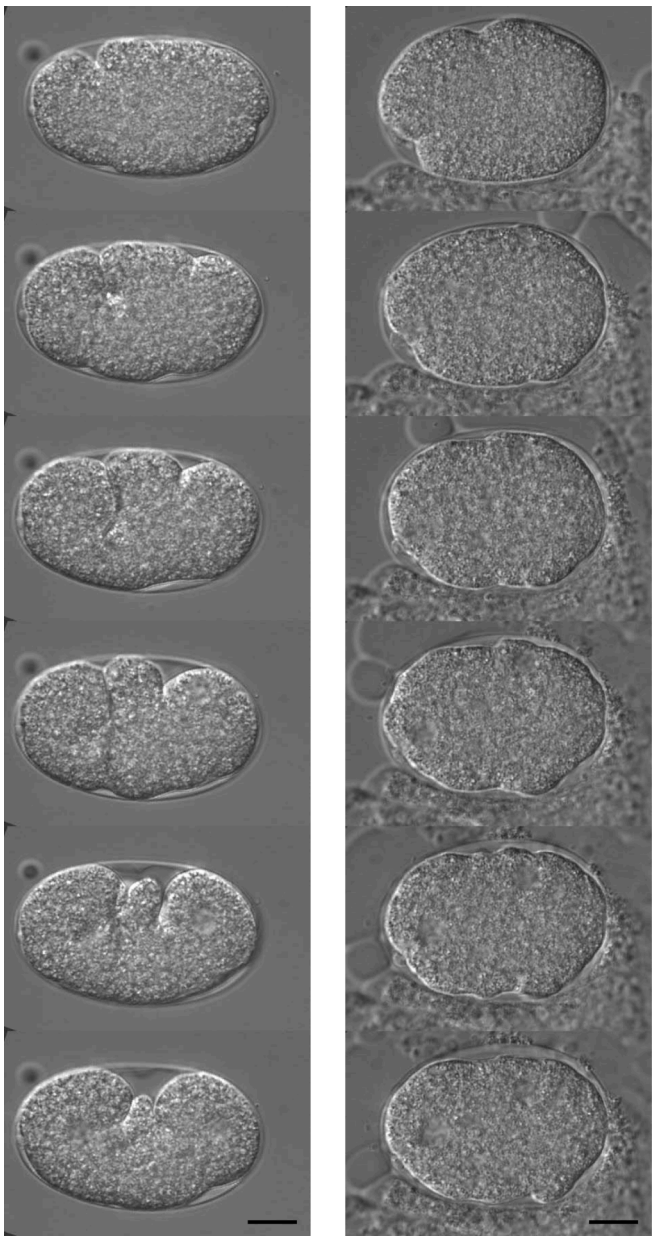


Figure 19: Time lapse recordings of N2 (left panel) and CX51 dyn-1(ky51ts) (right panel) embryos. First picture shows the embryo directly after the shift to 31°C. The following five pictures were taken in two minute intervals after the shift. Bar, 10µm.

3.6.1.2 Expansion of posterior domain

As a comparison, the same types of embryos were also recorded when most of the posterior domain had already formed, to see if dynamin is needed for the progression of the size of the posterior domain.

Wild type and dyn-1(ky51ts) embryos were used for the time-lapse recordings, but the embryos were shifted to 31°C at a later time point, when the posterior domain had already reached more than 30% of the whole embryo (Figure 20).

When shifted later to 31°C the situation looked different than at room temperature. The wild type as well as the mutant could continue extending the smooth, posterior domain. The embryos that were chosen for this experiment had already established most of the posterior domain before the shift (more than 30% of the whole embryo). The embryos did not look completely the same. The dyn-1(ky51ts) mutant seemed to show weaker ruffling and not such a big pseudocleavage furrow as the wild type. The pseudocleavage furrow in dyn-1(ky51ts) embryos also seemed to regress earlier than normal, even before the two pronuclei met (Figure 20).

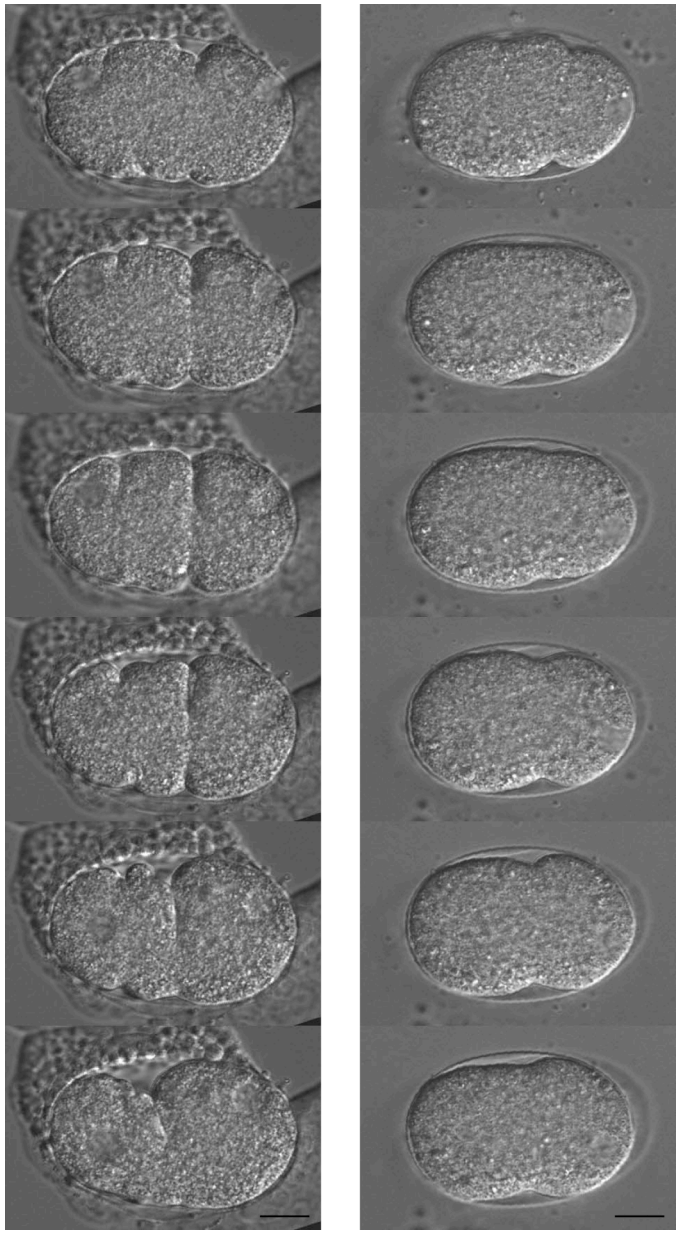


Figure 20: Time lapse recordings of N2 (left panel) and CX51 dyn-1(ky51ts) (right panel) embryos. The first picture shows the embryo directly after the shift to 31°C. The temperature shift was performed, when polarity was already mostly established (posterior domain covers already more than 30% of the embryo). Bar, 10µm.

These results indicate that dynamin is needed for the initiation of polarity establishment, but later on the posterior domain can expand without dynamin.

The next step was to determine at what stage of polarity establishment dynamin becomes dispensible by analysing all the time lapse recordings made with N2 and CX51 dyn-1(ky51ts) embryos, in which the shift to the restrictive temperature was made a different times.

To visualize the results of all the recordings at room temperature and 31°C I made a graph showing the results of each single embryo recorded.

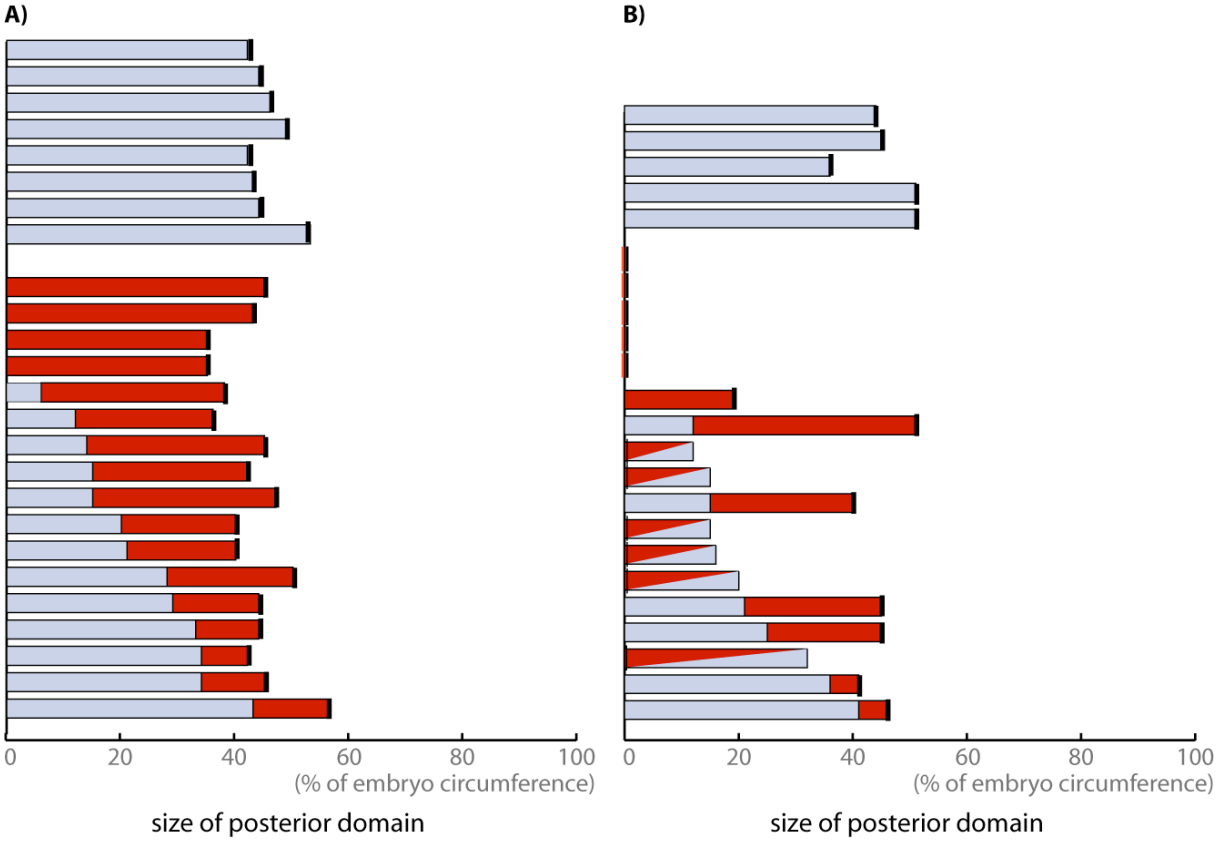


Figure 21: Size measurements of the posterior domain of CX51 dyn-1(ky51ts) (B) and N2 (A) embryos using time-lapse recordings. Recordings were taken at RT (blue) and 31°C (red) with temperature shifts at different time points during polarity establishment, in the x-axis the percentage of the posterior domain compared to the whole circulmference of the embryo had been calculated. Blue bars represent the controls, without shift to 31°C. Bars that are half blue and half red (diagonal) show embryos that could establish polarity until the time point of the shift and then regressed the posterior domain again after the shift. Black lines indicate the size of the posterior domain at the end of the recordings.

Therefore, I calculated the percentage of the posterior domain of each embryo compared to the total circumference of the whole embryo. This I calculated at two time points for each

embryo, first right after the shift to 31°C and then, when the posterior domain reached the maximum size before the posterior pronucleus started moving toward the anterior one, or when the posterior domain had regressed completely again. With that it was possible to show an increase or a decrease of the size of the posterior domain.

There was always an increase of the size of the posterior domain in the wild type (Figure 21.A), even when the embryo was shifted to 31°C before the initiation of polarity establishment. In contrast, in the mutant the temperature shift lead to the regression of the posterior domain quite often. Embryos that were shifted to the restrictive temperature before the initiation of polarity establishment could not establish polarity at all (Figure 21.B).

This is again an indication that dynamin is needed for the initiation of polarity establishment and for some time during the extension of the posterior domain. It was not possible to determine precisely when dynamin is no longer required for polarity establishment.

3.7 Does dynamin affect centrosome assembly?

Centrosomes are required for the initiation of polarity establishment, but don't seem to be required for the expansion and maintenance of the posterior domain. The function is similar to the function that dynamin seems to have.

It was also interesting to determine if the lack of dynamin had any effect on centrosome assembly during the early phase of polarity establishment.

I decided to take α-tubulin as a marker for the centrosomes in early polarity establishment. I looked at α-tubulin in dynamin mutants during polarity establishment at the restrictive temperature to determine if centrosome assembly or function was affected.

The one-cell embryos were used for time-lapse fluorescent imaging at room temperature and 31°C. Recordings started during early polarity establishment and the samples, that were recorded at 31°C were immediately shifted to the restrictive temperature. The α tubulin::YFP control was treated the same way and both strains were also recorded at room temperature without any shift to serve as controls.

The centrosomes appeared as small bright dots close to the male pronucleus. The embryos were recorded very early during polarity establishment (Figure 22, right side: α-tubulin::YFP, control; left side: dyn-1(ky51ts); α-tubulin::YFP, mutant).

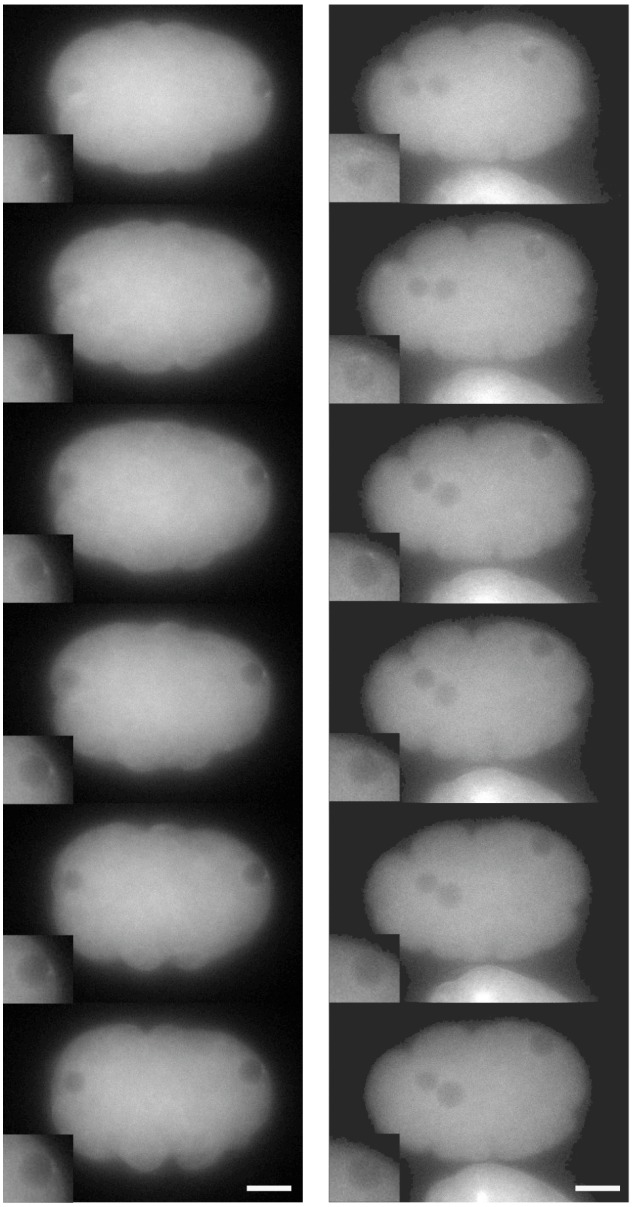


Figure 22: time lapse recordings of αtubulin::YFP (right panel) and dyn-1(ky51ts); αtubulin::YFP (left panel) at 31°C. The first picture was in both cases the first image taken at 31°C; the following ones were taken in 1min intervals after the shift to 31°C. The squares in the left bottom corner in each picture show a blow up of the region around the centrosome. In the mutant, there are two female pronulei next to each other on the right half of the embryo. There might have been a problem in polar body extrusion, but this has no effect on centrosome maturation. Bars, 10µm. (α tubulin::YFP: n=1 RT, n=3 31°C; dyn-1(ky51ts);α tubulin::YFP: n=1 RT, n=2 31°C)

It was quite hard to record the centrosomes at this early stage using α-tubulin::YFP as a marker. The centrosomes were very small and they moved a lot, meaning that focusing was hard and the centrosomes were out of focus quite easily. No difference between the mutant and the control could be determined. This may tell us that there is no impairment in

centrosome assembly in the dyn-1(ky51ts) mutant at the restrictive temperature when shifted during expansion of posterior domain. However, the dyn-1(ky51ts) mutants examined could all establish polarity because the shift occurred at a stage where dynamin is not essential for the expansion of the posterior domain, so it still remains to be determined what happenes to the centrosomes when the embryo cannot establish polarity at all or regresses the posterior domain after the shift to the restrictive temperature.

3.8 Does dyn-1 have an effect on NMY-2 distribution?

There is a requirement for acto-myosin contractility to achieve proper asymmetry in the embryo. NMY-2 distributes asymmetrically to the anterior side upon polarity establishment. At this point I was interested if the loss of dyn-1 function has an effect on NMY-2 distribution at an earlier phase of asymmetric cell division, during expansion of the posterior domain.

Therefore dyn-1(ky51ts);NMY-2::GFP embryos at room temperature and the restrictive temperature were observed.

Again NMY-2::GFP control and dyn-1(ky51ts);NMY-2::GFP mutant strains were used. The shift to 31°C was performed during an early phase of polarity establishment and the images were taken until the end of polarity establishment. This allowed me to look at the earlier stage of asymmetric cell division.

In the wild type, NMY-2 distributed asymmetrically to the anterior side of the embryo. Also at 31°C the NMY-2::GFP embryo, which served as a control, showed this effect. However the mutant dyn-1(ky51ts);NMY-2::GFP showed a very similar distribution pattern of NMY-2 (Figure 23). The same effect could be observed in the later stage of asymmetric cell division, shown in Figure 16. No significant differences in NMY-2 distribution could be determined between the control and the mutant.

This brings me again to the conclusion that dynamin function has no effect on NMY-2 distribution after the initiation of polarity establishment and during expansion of the posterior domain. The dyn-1(ky51ts) mutant embryos examined established polarity and could continue to expand the posterior domain after the shift. Further experiments will hopefully show what happens in the case of dyn-1(ky51ts) mutants in which the posterior domain regresses or in which polarity is not established at all.

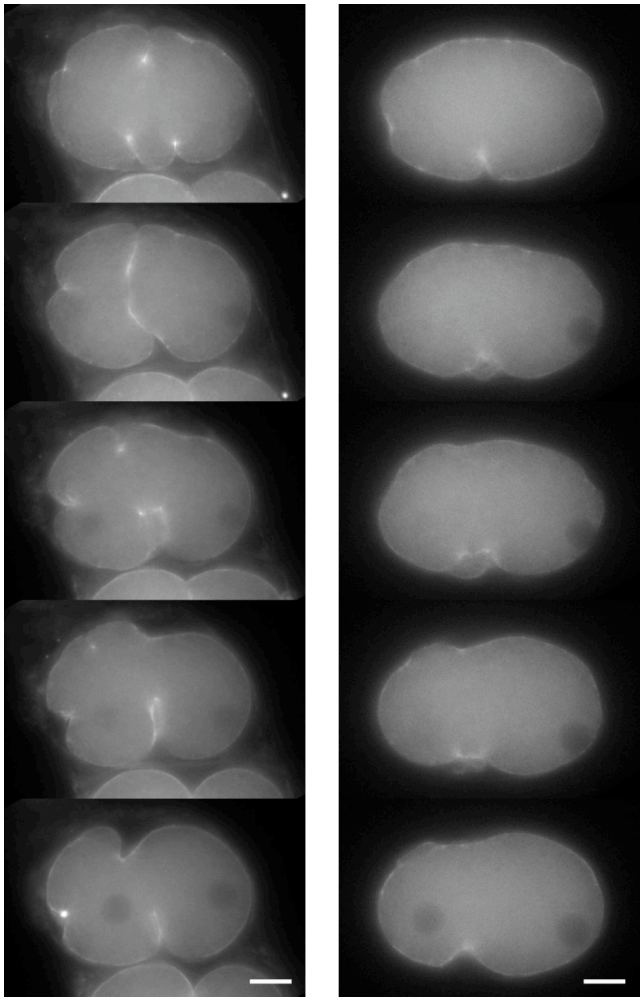


Figure 23: time lapse recordings of NMY-2::GFP (488nm) at 31°C. The images were taken in an interval of 2 minutes. NMY-2::GFP is shown the left panel the mutant dyn-1(ky51ts);NMY-2::GFP in the right one. The first images in both panels, are the first ones taken after the temperature shift. Bars, 10µm. (NMY-2::GFP: n=1 RT, n=1 31°C; dyn-1(ky51ts);NMY-2::GFP: n=1 RT, n=2 31°C).

3.9 Is PAR-3 distribution affected in the dyn-1(ky51ts) mutant?

Before polarity establishment there is ruffling of the whole embryo, which is due to contractions of the acto-myosin network across the whole embryo. After symmetry breaking the acto-myosin contractility regresses to the anterior half of the cell. PAR asymmetry follows this event. It was interesting to know how the PAR proteins are distributed in the temperature sensitive dyn-1(ky51ts) embryos, where contractile asymmetry was either not established or not maintained. Is PAR asymmetry also lost?

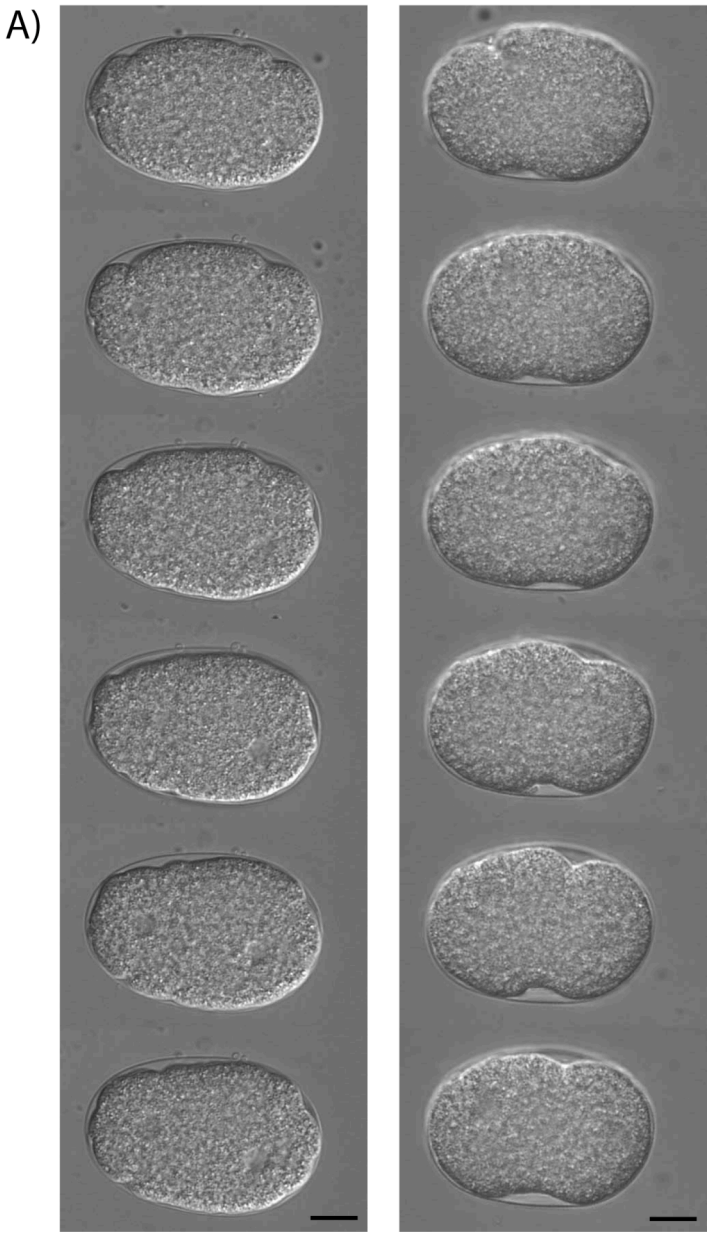
3.9.1 Time lapse recordings

The first of the PAR proteins that I looked at was PAR-3. It turned out that the GFP signal in the dyn-1(ky51ts);PAR-3::GFP strain was too weak to get any useful results. But there was

another thing that seemed to be interesting about this strain. The dyn-1(ky51ts) temperature sensitive effect on expansion of the posterior domain seemed to be enhanced in PAR-3::GFP embryos.

I therefore looked in more detail at the size threshold for posterior domain expansion in dyn-1 mutants expressing PAR-3::GFP.

I obtained the dyn-1(ky51ts);PAR-3::GFP strain through a cross between CX51 dyn-1(ky51ts) and PAR-3::GFP. This strain was then used for time-lapse recordings in which the embryos were shifted to 31°C at different time points during polarity establishment. The same experiment was made with PAR-3::GFP as control.



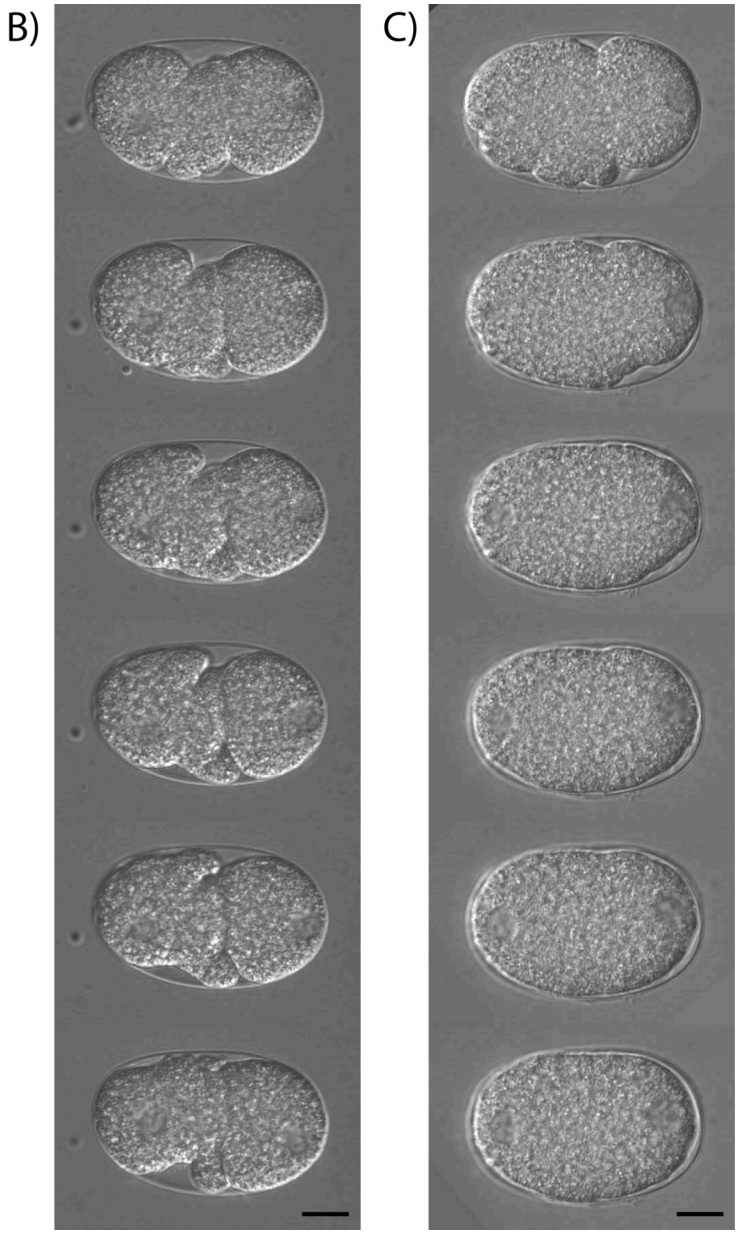


Figure 24: Time lapse recordings of dyn-1(ky51ts) and dyn-1(ky51ts);PAR-3::GFP embryos. In all cases the posterior domain has already reached a size of more than 15% of the whole embryo at the time of the shift from room temperature to 31°C. The first picture shows the embryo immediately after the shift to 31°C, the following pictures were taken in one-minute intervals. A) CX51 dyn-1(ky51ts), B) PAR-3::GFP and C) dyn-1(ky51ts);PAR-3::GFP embryos. Bar,10µm.

In the CX51 dyn-1(ky51ts) embryos (Figure 24, A) polarity could establish without the function of dyn-1 if the posterior domain had already reached a certain size. The exact time point until dyn-1 is needed could not be determined. In the PAR-3::GFP and dyn-1(ky51ts);PAR-3::GFP embryos (Figure 24, B and C) the posterior domain had also already reached more than 15% of the embryo. While the smoothing of the cortex could proceed after the shift to 31°C in the PAR-3::GFP control, the posterior domain completely regressed in the

dyn-1(ky51ts);PAR-3::GFP mutant. Thus in the dynamin mutant in the presence of PAR-3::GFP the posterior domain seemed more likely to regress than in the dynamin mutant alone

I calculated the percentage of the posterior domain of each embryo (like in section 3.6.1.) compared to the total circumference of the whole embryo. This I calculated at two time points for each embryo, first right after the shift to 31°C and then, when the posterior domain reached the maximum size until pronuclear movement from the cortex or when the smooth domain had completely regressed. With that it was possible to show an increase or a decrease of the size of the posterior domain.

The PAR-3::GFP control embryos showed a comparable average size of the posterior domain at room temperature as the N2 wild type (Figure 21). But differences already started to show in the control at 31°C. After the shift the posterior domain nearly stopped expanding in most cases (Figure 25, A). In the dyn-1(ky51ts);PAR-3::GFP mutant, the effect was even more severe. Already at room temperature the average size of the posterior domain was lower than in the experiment with CX51 dyn-1(ky51ts) (Figure 21), where it was around 45%. At 31°C the majority of the embryos could not continue expanding the posterior domain. Quite the contrary happened: the posterior domain regressed almost in all cases, even when already nearly half of the embryo showed smoothing of the cortex at the time of the shift.

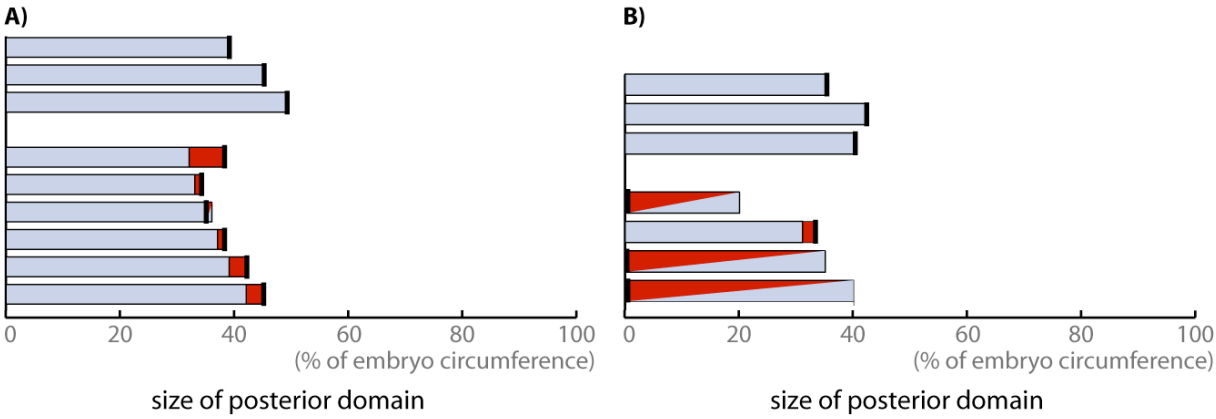


Figure 25: Size measurements of the posterior domain of dyn-1(ky51ts);PAR3::GFP (B) and PAR3::GFP (A) embryos using time-lapse recordings. Recordings were taken at RT (blue) and 31°C (red) with temperature shifts at different time points during polarity establishment. Blue bars represent the controls without temperature shift. Half blue, half red bars (diagonal) symbolize a decrease of the size of the posterior domain after the shift to 31°C. Black lines indicate the size of the posterior domain at the end of the recordings.

These results together indicate that the temperature sensitive mutant of dyn-1 seems to be even more important for expansion of the posterior domain in the presence of GFP-PAR-3. It

is possible that GFP-PAR-3 represents overexpression of PAR-3, suggesting that the balance of anterior and posterior PAR proteins may be important, especially when dynamin function is affected. I thought it would be interesting to take a closer look at this question with further experiments.

3.10 Is there a similar effect visible in PAR-2 RNAi?

PAR-2 is a posterior PAR protein. It is opposite to the anterior PAR protein PAR-3. However it is not required for polarity establishment, because the acto-myosin network and PAR-3 and PAR-6 are localized to the anterior side of the embryo even without it, but it is required for the maintenance of PAR asymmetry after pseudocleavage.

3.10.1 Time lapse recordings

As the dyn-1 temperature sensitive mutant showed a more severe phenotype in combination with PAR-3::GFP, the question arose if this effect would also be seen in the (partial) knock-down of PAR-2. In PAR-3::GFP embryos, an anterior PAR-protein is over-expressed. So what happens if instead of the up-regulation of an anterior PAR-protein, the ratio is shifted into the same direction by decreasing the expression of a posterior PAR-protein with RNAi? In this case the situation could be tested in a more controlled manner.

A PAR-2 RNAi experiment, with the temperature sensitive mutant and the wild type at two different time points was made.

I transferred N2 wild type worms and CX51 dyn-1(ky51ts) worms to par-2 RNAi plates (see section 2.9.2) and made time-lapse fluorescent images after 24 and 48 hours. Until recording, the plates were incubated at 16°C. I started recording at 31°C sometime during polarity establishment and continued until the end of polarity establishment. The same procedure was performed with N2 worms on NGM/OP50 plates as a control. The second control was N2 on the RNAi plates and on NGM/OP50 plates only at room temperature without, temperature shift.

To get a better impression of the results together I also quantified all the results from the par-2 (RNAi) experiments at 24 and at 48 hours.

To visualize the results of all the recordings at room temperature and 31°C I made a graph showing the results of each single embryo recorded.

Therefore I calculated the percentage of the posterior domain of each embryo compared to the total circumference of the whole embryo (like in section 3.6.1.). This I calculated at two time points for each embryo, first right after the shift to 31°C and then, when the posterior domain reached the maximum size. With that it was possible to show an increase or a decrease of the size of the posterior domain.

In the wild type at room temperature, the size of the posterior domain could develop nearly to the full size. With a shift to 31°C it looked a bit different: it seemed that after the shift, the posterior domain could not expand to the full size any more, at least when the shift occurred before the posterior domain had reached a size of less than 30% before the temperature shift (Figure 28, A). Nevertheless the posterior domain in the control embryos could always establish at 31°C, with 24 and at 48 hours par-2 RNAi (Figure 26, left panel: N2 embryo after 48h par-2 RNAi treatment).

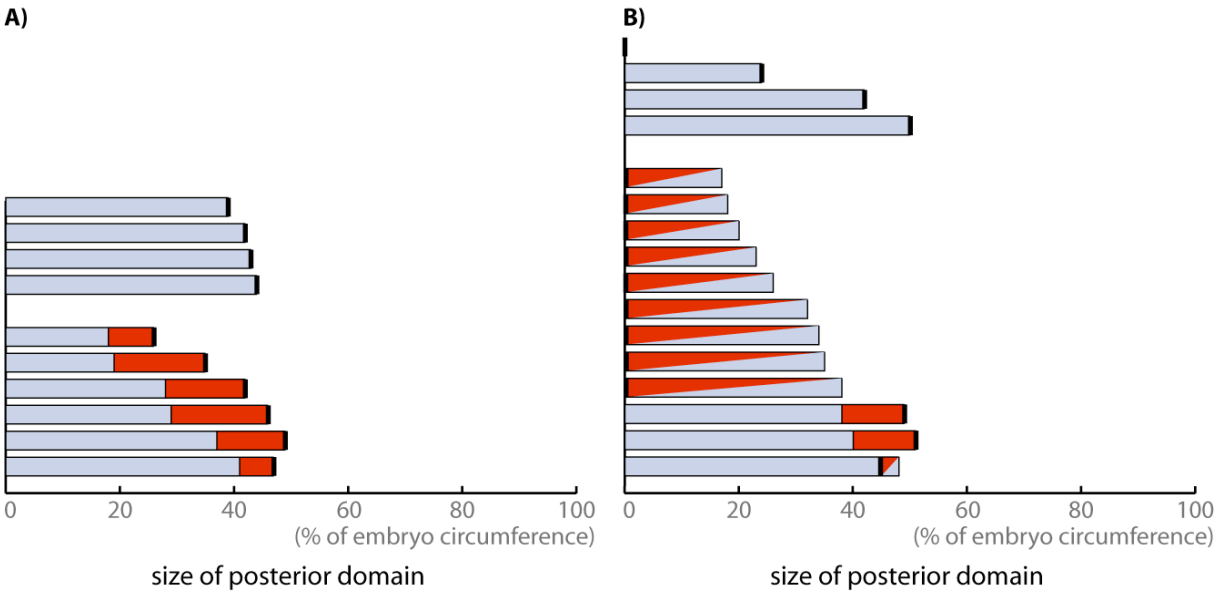


Figure 26: Size measurements of the posterior domain of par-2 (RNAi) for 24 hours performed with N2 (A) and CX51 dyn-1(ky51ts) (B) embryos using time-lapse recordings. Recordings were taken at room temperature (blue) and 31°C (red) with temperature shifts at different time points during polarity establishment. Blue bars represent the controls without temperature shift. Half blue, half red bars (diagonal) symbolize a decrease of the size of the posterior domain after the shift to 31°C. Black lines indicate the size of the posterior domain at the end of the recordings.

In the dyn-1(ky51ts) mutant the phenotype was again more severe with par-2 (RNAi) than without. In the dyn-1(ky51ts); par-2 (RNAi) embryos at room temperature, the posterior domain could not reach the average size of 45% in all cases. In the dyn-1(ky51ts); par-2 (RNAi) embryos that were shifted to 31°C, the posterior domain regressed completely in

almost all cases. The posterior domain was only able to continue to expand when it had already reached a size of around 40% before the shift (Figure 26, B, Figure 28 top picture, right panel).

After 48 hours of par-2 RNAi both the wild type and the dyn-1(ky51ts) controls without temperature shift looked similar to those at 24 hours of par-2 RNAi (Figure 28, bottom picture, left panel). In some cases, however, the posterior domain of the mutant even regressed when the embryo was recorded at room temperature after 48h of par-2 RNAi (Figure 28, right panel). N2 worms depleted of PAR-2 by RNAi at 31°C showed comparable phenotypes to the ones at 24h (Figure 29). The mutant with the temperature shift to 31°C showed a difference. The embryos completely failed to continue establishing polarity until they had reached a size of more than 40% before the shift. In all other cases the posterior domain regressed completely after the shift to the restrictive temperature.

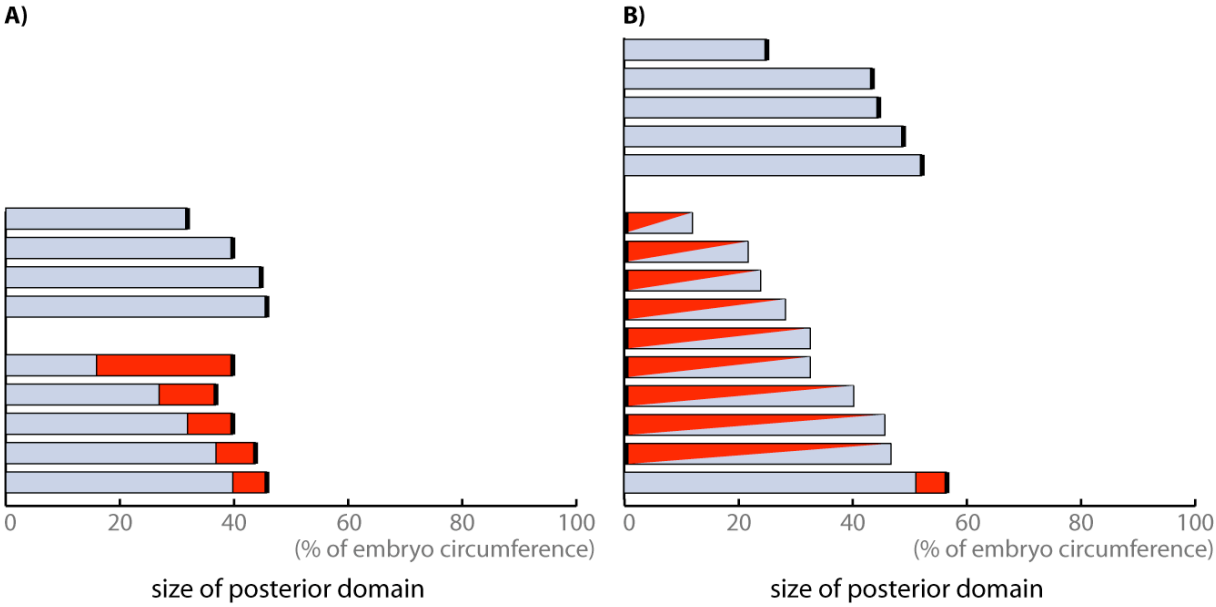


Figure 27: Size measurements of the posterior domain of par-2 (RNAi) for 48 hours performed with N2 (A) and CX51 dyn-1(ky51ts) (B) embryos using time-lapse recordings. Recordings were taken at room temperature (blue) and 31°C (red) with temperature shifts at different time points during polarity establishment. Blue bars represent the controls without temperature shift. Half blue, half red bars (diagonal) symbolize a decrease of the size of the posterior domain after the shift to 31°C. Black lines indicate the size of the posterior domain at the end of the recordings.

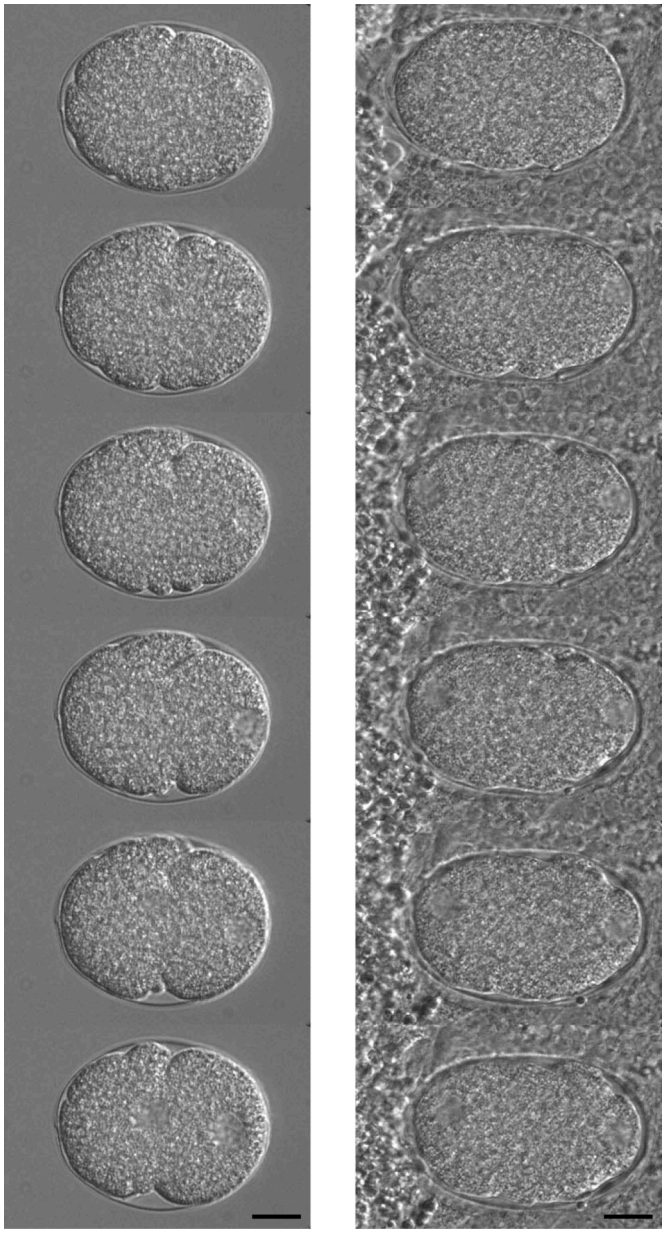


Figure 28: Time lapse recordings of N2 and CX51 dyn-1(ky51ts) embryos. The first picture (left panel) shows a par-2 (RNAi) embryo, a N2 embryo that has been on a par-2 RNAi plate for 48h and then has been shifted to 31°C during polarity establishment. The embryo in the right panel is CX51 dyn-1(ky51ts), which has spent 24h on a par-2 RNAi plate before recording. It has also been shifted to 31°C during polarity establishment. First picture in both cases was taken directly after the shift, the following ones were taken in one-minute intervals from the shift on. Bar,10µm.

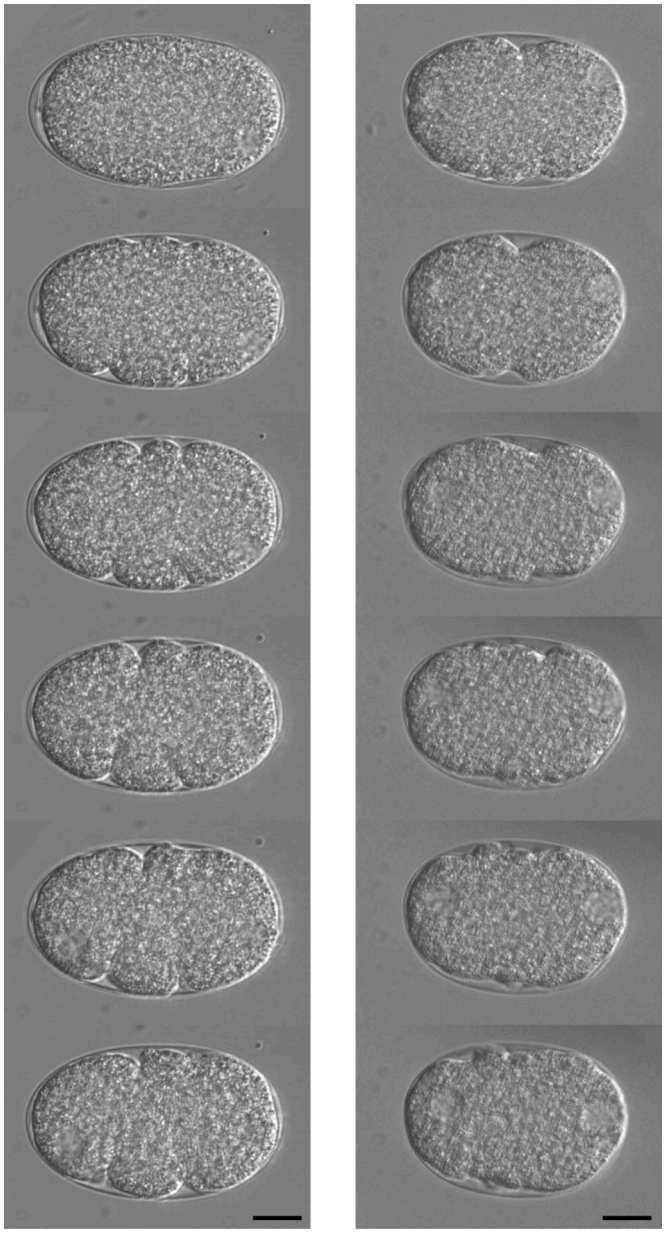


Figure 29: Time lapse recordings of CX51 dyn-1(ky51ts) embryos. On the left there is an embryo, which had spent 24h on the par-2 (RNAi) plate, the embryo on the right side was on the par-2 RNAi feeding plate for 48h. Both embryos were not shifted to 31°C during recording, but only recorded at room temperature. First picture in both cases was taken directly after the shift, the following ones were taken in one-minute intervals from the shift on. Bar,10µm.

All in all it seems as if the par-2 RNAi on the embryos shows similar effects on the polarity establishment as the PAR-3::GFP. This suggest, that par-2 RNAi has an influence on polarity establishment in CX51 dyn-1(ky51ts) embryos. Polarity establishment seems to be more sensitive to the shift to the high temperatures in dyn-1(ky51ts); par-2 (RNAi) embryos, which could mean that they are more dependent on the dyn-1 function if par-2 is partially or even

fully knocked down. It could mean that DYN-1 and PAR-2 work in parallel pathways during polarity establishment.

3.11 Where are components of the vesicle transport pathway localized during polarity establishment?

The aim was to get a better understanding of how dynamin contributes to polarity establishment. Therefore it was also interesting to look at different components of the intracellular vesicle transport during polarity establishment in wild type worms. This analysis might suggest whether there are certain trafficking pathways that exhibit an asymmetric distribution, which might suggest a link to polarity establishment.

3.11.1 Endoplasmic reticulum

The endoplasmic reticulum plays an important role in vesicle transport, so I decided to take SP12, a signal peptidase, as an ER marker.

I used the SP12::GFP strain to make time lapse recordings during polarity establishment and pronuclear meeting.

The GFP signal for the protein SP12 seems to localize in stringy patterns all over the cell, especially around the pronuclei and later on around the centrosomes and the mitotic spindle. I could not recognize any asymmetry in the distribution pattern throughout the polarity establishment until cytokinesis (Figure 30).

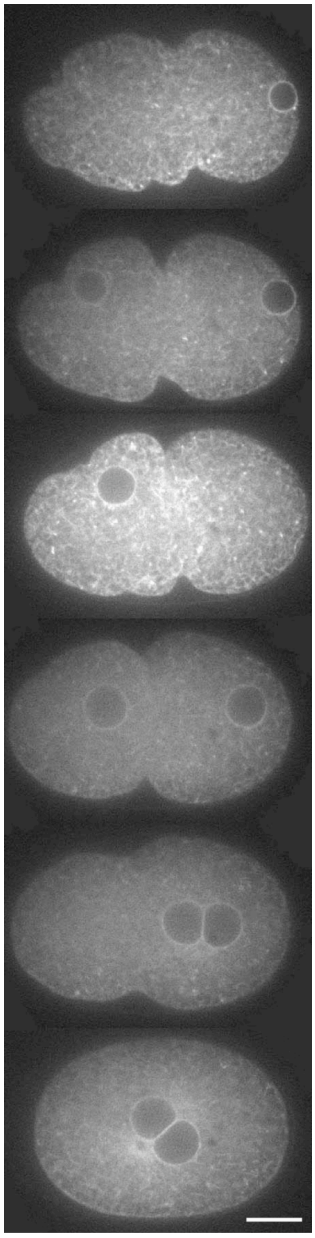


Figure 30: time lapse recordings of an SP-12::GFP embryo at room temperature. The pictures were taken in 2 minute intervals. Bar, 10µm. (n=8)

3.11.2 Exocyst complex

Secondly I looked at the exocyst complex with the marker protein EXOC-7, an exocyst component protein, to observe its subcellular localisation.

I generated an EXOC-7::GFP worm strain and I recorded embryos from polarity establishment until after pronuclear meeting.

For the generation of the EXOC-7::GFP strain the construct was cloned and later bombarded into DP38 worms. The worms were selected for a GFP signal. The worms containing the

GFP-tagged protein could be used for fluorescent time-lapse imaging. The recordings started during polarity establishment and were stopped at some point after pronuclear meeting. The 100x objective and the spinning disc were used for imaging. All the recordings were made at room temperature.

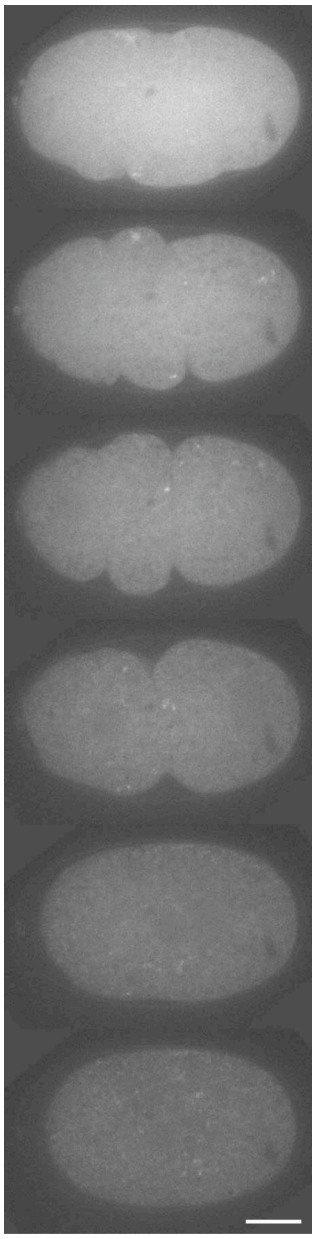


Figure 31: time lapse recordings of an EXOC-7::GFP embryo at room temperature. The pictures were taken in 2 minute intervals. Bar, 10µm. (n=4)

Throughout the whole recording no specific pattern of the EXOC-7::GFP signal could be recognized. The bright dots that appeared at seemingly random places could also be protein aggregates (Figure 31). For that reason further studies on this protein, e.g. in combination

with the dynamin temperature sensitivity were not useful, because it would have been impossible to detect changes in the protein distribution in the absence of dyn-1 function.

3.11.3 Early endosomes

It has already been established that RAB-5 is an appropriate marker of early endosomes. How do the early endosomes localize in the cell?

I used RAB-5::GFP strain for time lapse imaging. For the recordings I took the 100x lens.

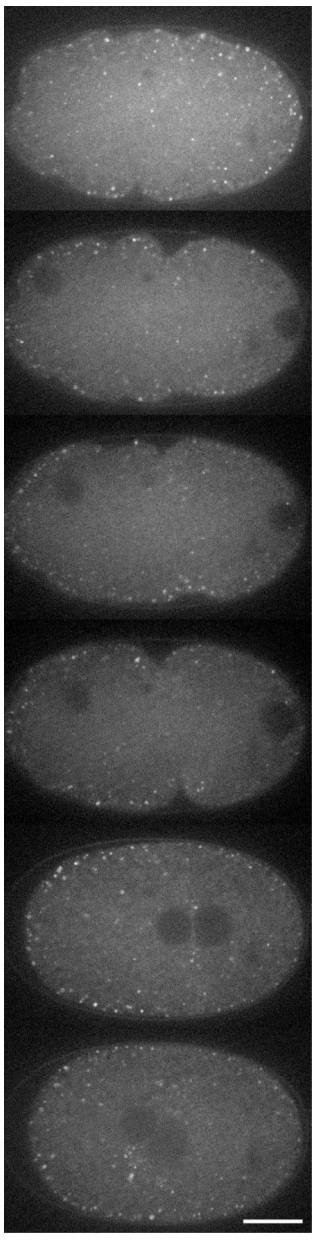


Figure 32: time lapse recordings of an RAB-5::GFP embryo at room temperature. The pictures were taken in 2 minute intervals. Bar, 10μm. (n=3).

Therefore I used the RAB-5::GFP line. All recordings were performed at room temperature with the 100x lens, with spinning disk illumination. I started imaging at some point during polarity establishment.

The signal appeared as bright dots. They were first distributed equally across the cell, close to the cortex, but with polarity establishment the early endosomes seemed to be translocated to the anterior part of the embryo. The endosomes were located at the anterior side and at the pseudocleavage furrow (Figure 32).

3.12 syn-4 RNAi

Dynamin is involved in endocytosis and cytokinesis (Thompson et al., 2002). According to the results so far it also seems to be required for polarity establishment. On the other hand it has been reported that endocytosis is not required for polarity establishment. A paper had been published by Poteryaev (Poteryaev et al., 2007) where they knocked down RAB-7, a small GTPase required for endocytic trafficking, by RNAi. Polarity can still be established. So it may be a different function of dynamin that is necessary for polarity establishment. What is the dynamin-dependent event in polarity establishment?

One other possibility would be that it has something to with vesicle targeting to the plasma membrane. Syn-4 is important for this process and, as a result, it is essential for cytokinesis in *C. elegans*. How does the cell behave during polarity establishment when syn-4 is kocked down? Does syn-4 (RNAi) have a similar effect on polarity establishment as the reduction in dynamin activity?

To answer this question I treated N2 worms with syn-4 double stranded RNA. After 41 hours the embryos were ready to be recorded. Images were taken with the 40x lens at room temperature.

The worms got the RNAi through feeding. Therefore I made syn-4 RNAi feeding bacteria and later feeding plates. The worms were transferred to the plates 41 hours before recording. The images I took with the 40x lens and wide field illumination. I started recording at some point during polarity establishment until cytokinesis. N2 worms that were kept on NGM/OP50 plates in parallel were used as a control.

The N2 embryos treated with the syn-4 RNAi still could establish polarity, even though the posterior domain seemed to be smaller than in the control embryos. The cell cycle from

polarity establishment until cytokinesis appeared to take significantly longer than in wild type. Apart from this the syn-4 RNAi embryos could not undergo cytokinesis (Figure 33).

The results agree with the results with had been pusblished earlier (Jantsch-Plunger and Glotzer, 1999), where syn-4 RNAi was applied to the embryos by injection, and in which it was shown that syn-4 RNAi embryos cannot complete cytokinesis. I could therefore observe multinucleated cells. They seem to have osmotic problems and the cytoplasm is sometimes very granulated. As the embryos still can establish the posterior domain, I can conclude that syn-4 is not required for polarity establishment.

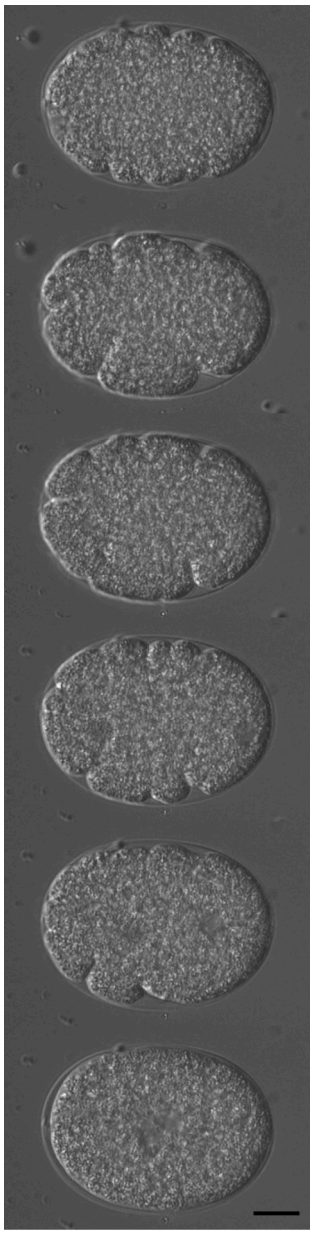


Figure 33: time lapse recordings of a N2 embryo that had been treated with syn-4 (RNAi) for 41 hours. Recordings are all the time at room temperature. The pictures were taken in 4-minute intervals. Bar, 10µm. (n=3/4)

3.13 Inhibitor studies

Next I wanted to know if the effect of dynamin on polarity establishment is related to the transport of vesicles through the endoplasmic reticulum.

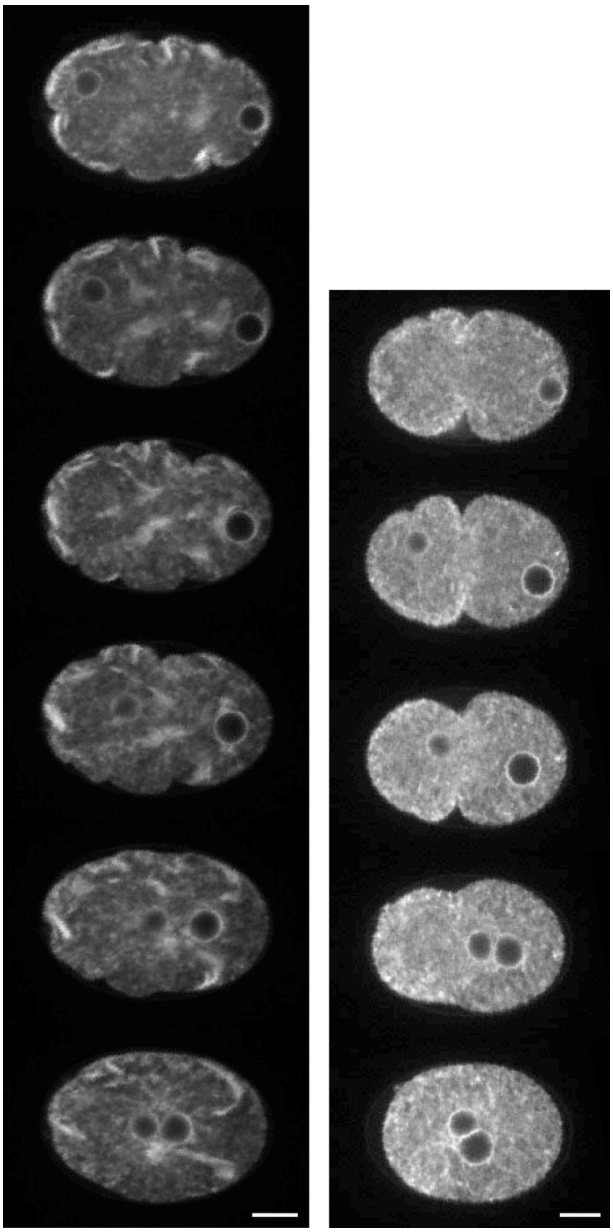


Figure 34: time lapse recordings of an SP12::embryo treated with 2µg/ml Brefeldin A for 20 minutes. All images were taken at room temperature. The 40x lens and spinning disc illumination were used for fluorescent time lapse imaging. The pictures were taken in 2 minute intervals. Bar, 10µm. (n=7)

The idea of the experiment was to block the vesicle transport from the endoplasmatic reticulum through the inhibitor Brefeldin A (Fujiwara et al., 1988). Embryos were assessed for polarity establishment by time-lapse microscopy after treatment with Brefeldin A.

I used worms expressing the endoplasmic reticulum marker SP12::GFP to confirm that the drug treatment worked. Brefeldin A causes the collapse of the structure of the endoplasmic reticulum (Fujiwara et al., 1988). The worms were put into a solution containing 2µg/ml Brefeldin A and the tail was cut off, so that the inhibitor could reach the embryos. After 20 minutes the worms were dissected and the embryos could be used for imaging. For this experiment I used the 40x lens with spinning disc illumination. I started recording at some point during polarity establishment until after pronuclear meeting. SP12::GFP embryos without inhibitor treatment served as a control.

From the beginning on it was clearly visible, that the embryos treated with the Brefeldin A inhibitor had a significantly distinct endoplasmic reticulum distribution, compared to the control. Big clusters of SP12::GFP were dispersed throughout the cytoplasm without any specific pattern (Figure 34, left panel). In the control the ER seemed to be more evenly distributed all over the cell (Figure 34, right panel). Although there was this severe phenotype of the SP12::GFP in the cytoplasm, the embryos could still establish polarity.

This result could indicate that the effect of dynamin on polarity establishment is not due to the vesicle transport from the endoplasmic reticulum. It must be a different function or a combination of functions of dynamin that in its absence causes this defect in polarity establishment.

4 Discussion

Through experiments with the temperature sensitive dyamin mutant I could confirm again that dynamin is necessary for cytokinesis. Without the function of dynamin, cell division could not be completed. I was interested in the in the effect of the loss of dynamin function on NMY-2 distribution. NMY-2 plays a role in anterior-posterior polarity and cytokinesis (Guo and Kemphues 1996). According to the results it seemed as if there was no significant difference between the dynamin mutant at the restrictive temperature and the wild type. In the brightfield recordings the spindle seemed to be misslocated, so I checked with α-tubulin::GFP, but the phenotype appeared in both the mutant and the wild type at the restrictive temperature. When I looked at the early endosomes, I could see a difference between wild type and mutant at 31°C. As soon as the embryo was shifted to the higher temperature, the signal for endosomes seemed to decrease rapidly. It seemed as if number of vesicles got less after the shift to the restrictive temperature, which shows the importance of dynamin for early endosomes. This seems logical, because dynamin is required for clipping the vesicles off the plasma membrane in the clathrin-coated endocytosis pathway (McNiven et al., 2000).

After these initial experiments I could start to answer the main question: Is dynamin required during polarity establishment?

In experiments with CX51 dyn-1(ky51ts) (and N2 as control) it showed that embryos that have been shifted to the restrictive temperature before the initiation of polarity establishment could not even initiate polarity establishment. Embryos that were shifted shortly after the initiation showed the tendency to regress the smooth domain again. Only after the posterior domain had reached a certain size, the function of dyanmin seemed to be dispensable. Which time point this exactly is still remains to be determined. The distribution of NMY-2 was not affected also at earlier stages of polarity establishment and there was a strong indication that there was no problem with centrosome assembly at these time points. The next surprise came when I wanted to check for the PAR proteins in the temperature sensitive dynamin mutant. The line with dyn-1(ky51ts) and PAR-3::GFP suddenly showed a higher sensitivity to the restrictive temperature that dyn-1 alone, meaning that the posterior domain regressed even from a bigger size and that there were slight phenotypes already in the PAR-3::GFP embryos at 31°C and in the dyn-1ts(ky51ts);PAR-3::GFP at room temperature. Could the possible overexpression of PAR-3 and with this the shift of the ratios between anterior and posterior PAR proteins be responsible for this? To get information about this I knocked-down PAR-2,

which is a posterior PAR-protein, with RNAi in the temperature sensitive dynamin mutant. The idea was to shift the ratio of the PAR proteins into the same direction, but in a controlled manner. This time the amount of posterior PAR proteins would be lower, instead of a higher amount of anterior PAR protein. When I analyzed dyn-1(ky51ts); par-2 (RNAi) embryos, a similar phenotype to dyn-1(ky51ts); PAR-3::GFP embryos was observed. This was quite interesting, because it could be a remark for the contribution of PAR-2 and dynamin to parallel pathways. Further I looked at different components of the intracellular vesicle transport to observe their localization in wild type embryos. The only asymmetrically distributed endomembrane component that I analyzed were endosomes, however endosomes do not appear to be essential for polarity establishment (Poteryaev et al., 2007). Later I asked if syn-4 RNAi would give the same phenotype as the lack of dyn-1, because they are both responsible for cytokinesis and SYN-4 is also responsible for vesicle targeting, but it showed that SYN-4 may not be essential for polarity establishment. Finally I wanted to find out if the function of dynamin in the vesicle transport deriving from the ER is involved in the deficiency to establish cell polarity, but the results indicate that this process is not involved.

4.1 Dynamin and polarity establishment

Later on I could show that dynamin, besides its function in cytokinesis during asymmetric cell division, is also involved in polarity establishment. Dynamin has various functions in the cell but which function is responsible for the defect in polarity establishment?

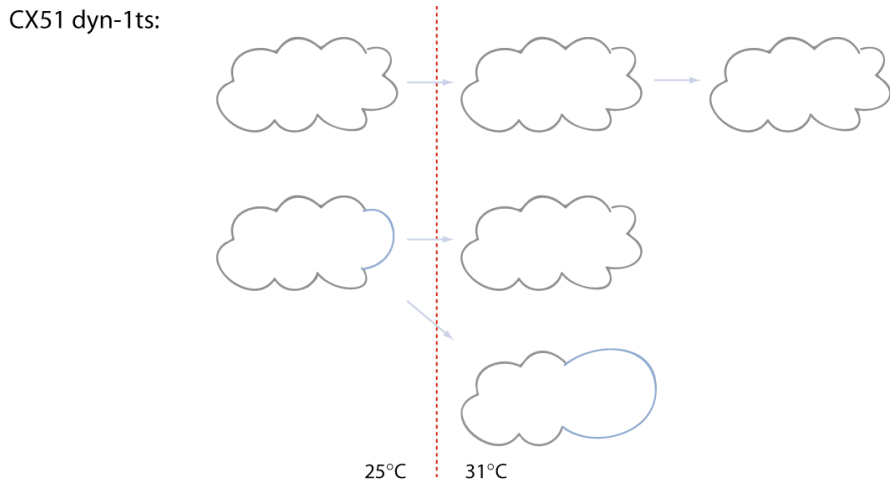


Figure 35: model of CX51 dyn-1(ky51ts) shifted to 31°C at different stages of polarity establishment. a) embryo shifted before the initiation of polarity establishment. b) embryo shifted after the initiation of polarity establishment. blue colour represents the smoothing of the cortex at the posterior domain.

Through observation of components of the vesicle transport with and without the function of dynamin and previous publications, some possible functions of dynamin could be ruled out with respect to dynamin's role in polarity establishment. In a paper where one-cell embryos had been treated with RAB-7 RNAi (Poteryaev et al., 2007) it showed that this does not influence polarity establishment. Through the depletion of RAB-7, which plays an important role in ealrly to late endosome transition (Poteryaev et al., 2007), the maturation of endosomes is blocked during receptor mediated endocytosis. From this it could be suggested that the endocytosis pathway is not required for polarity establishment. Could it be the opposite pathway from the ER to the plasma membrane? But also this possibility was ruled out through the block of the transport with the inhibitor Brefeldin A and RNAi of SYN-4. When treated with the inhibitor or depleted of SYN-4, the embryos could establish polarity. It remains to be determined what pathways are responsible for the polarity establishment defect observed in dynamin mutant embryos.

The acto-myosin network is required for polarity. Actin and myosin mutants do not have any cortical activity at all. dyn-1(ky51ts) embryos still have cortical activity so it is unlikely that dynamin impairs polarity establishment through the acto-myosin network. Consistently I didn't see any major problems with NMY-2::GFP in dyn-1(ky51ts) embryos. Centrosomes are required for polarity. Centrosome mutant embryos still have cortical activity, similar to dyn-1(ky51ts). Likewise centrosomes are only required for initiation of polarity but not for the expansion of the posterior domain. This is similar to dyn-1(ky51ts), but dyn-1(ky51ts) seems to also be required during expansion in many cases. Does dyn-1(ky51ts) affect the centrosomes? After polarity establishment, there is no detectable defect in microtubule nucleation, a centrosome function, in dyn-ts embryos. We still don't know if there is a problem if the embryos are shifted earlier.

Interestingly, dynamin appears to act in parallel to PAR-2 during polarization of the embryo, according to the results with PAR-3::GFP that most likely causes a PAR-3 overexpression and depletion of PAR-2 by RNAi. In one-cell embryos PAR-2 localizes to the posterior cell cortex during asymmetric cell division and is itself required for the polarisation of the embryo as well as unequal cleavage, differences in cell cycle length and spindle orientation (Cheng et al., 1995; Kirby et al., 1990). par-2 mutants are able to establish cortical polarity but not maintain asymmetry. The anterior PAR complex is initially segregated to the anterior half of the embryo in par-2 mutants but about midway through the cell cycle, the anterior PAR complex regresses into the posterior and cortical polarity is lost. Thus PAR-2 is essential for

the maintenance of polarity but appears to be dispensable for the initial establishment (Cuenca et al., 2003).

PAR-2 somehow excludes anterior PAR-proteins from the posterior side of the cell and enhances the association of PAR-1 and P granules to the posterior cortex (Boyd et al., 1996). The finding that par-2(RNAi);dyn-1(ky51) embryos are less capable of polarity establishment than either par-2(RNAi) or dyn-1(ky51) alone suggests an interaction between dynamin and PAR-2 pathways during polarity establishment. DYN-1 is a large GTPase, which is needed in endocytosis, synaptic vesicle recycling, cytokinesis and the engulfment of apoptotic cell corpse (Thompson et al., 2002). PAR-2 is predicted to be a RING finger-type ubiquitin E3 ligase (Boyd et al., 1996).

One possible way in which the PAR-2 and dynamin pathways interact is as follows:

Dynamin may support endocytosis of anterior PAR proteins to limit the size of the anterior PAR domain while PAR-2 may simultaneously restrict anterior PAR proteins from the posterior. If dynamin activity is reduced, the PAR-2 mediated restriction may be sufficient for the exclusion of anterior PAR proteins. However, if both dynamin activity and PAR-2 are reduced, the anterior PAR domain cannot be established. This model, however, would suggest that indeed the role of dynamin in polarity establishment is through its role in endocytosis. However, as discussed above, previous experiments showed that rab-7(RNAi) embryos, which are defective in endocytosis, can establish polarity. But if dynamin does have multiple functions during polarity establishment, it might be possible to see a role for endocytosis by combining rab-7(RNAi) with a reduction in PAR-2 function.

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6 Abbreviations

amp	ampicilin
n	Number of embryos observed
DNA	deoxyribonucleic acid
GAP	GTPase activating protein
GEF	guanidine exchange factor
GFP	green fluorescent protein
GTP	Guanidine triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
NGM	nematode growth medium
PCR	polymerase chain reaction
RNAi	RNA (Ribonucleic acid) interference
SNARE	soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor
tet	tetracyclin

Zum Abschluss möchte ich noch gerne die Leute erwähnen, die mich immer tatkräftig unterstützt und mir dadurch vieles erleichtert haben.

Ich danke vor Allem meiner Familie, im Besonderen meinen Eltern und meinem Bruder.

Weiters Carrie, Anne, Dominika, Sabina und Harue für die lehrreiche Zusammenarbeit und die gute Zeit.

Und natürlich meinen Freunden, die immer für mich da sind.

7 Curriculum Vitae

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Research Experience

Diploma Student	Research Institute of Molecular Pathology Vienna, AUSTRIA Dr. Carrie Cowan The role of dynamin during polarity establishment in *C. elegans* one-cell embryos	Oct. 2007 – present
Internship	Max F. Perutz Laboratories Medical University of Vienna Dr. Tim Skern Purification of foot-and-mouth disease virus leader protease and eukaryotic initiation factor 4E	Jul. 2007
Research Technician	Institute of Molecular Biotechnology Vienna, Austria Dr. Jürgen Knoblich	Oct. – Dec. 2006
Internship	Medical University Vienna Max F. Perutz Laboratories Department of Medical Biochemistry Dr. Karl Kuchler The role of different superoxide dismutases in *Candida albicans*	Nov. 2006

Internship	Max F. Perutz Laboratories University of Vienna Department of Microbiology and Immunobiology Dr. Pavel Kovarik	Jul. –Aug. 2006
Internship	Research Institute of Molecular Pathology Vienna, AUSTRIA Dr. Hartmut Beug	Apr. – Jun. 2006

Poster

Sophia Millonigg and Carrie R. Cowan. (2008). Research Institute of Molecular Pathology
"The role of dynamin during polarity establishment in one-cell embyos"
Poster presentation at the European Worm Meeting, Carmona, Spain

Languages

German
English
Spanish (basics)